PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Bert VOGELSTEIN *et al.*	)	Group Art No. 1645
	)	
Serial No: 09/784,305	)	Examiner: TBA
	)	
Filed: February 16, 2001	)	Docket No. 01107.00103

For: GENOME-BASED PERSONALIZED MEDICINE

PETITION TO EXPUNGE INFORMATION UNDER 37 C.F.R. § 1.59

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants have filed an Information Disclosure Statement in the application referenced above, citing four co-pending applications:

Serial No. 60/158, filed October 8, 1999;

Serial No. 09/461,047, filed December 15, 1999;

Serial No. 09/504,860, filed February 16, 2000; and

PCT/US00/27508, filed October 6, 2000.

Applicants respectfully request that, upon allowance of the present application, each of the U.S. applications be expunged from the application file and returned to the undersigned. Expungement is necessary to preserve the confidentiality of these applications.

Please charge the $130.00 fee under 37 C.F.R. § 1.17(h) to our Deposit Account No. 19-0733.

Respectfully submitted,

Date: July 25, 2001

By: Lisa M. Hemmendinger
Lisa M. Hemmendinger
Registration No. 42,653

Banner & Witcoff, Ltd.
1001 G Street, N.W., Eleventh Floor
Washington, D.C. 20001-4597
(202) 508-9100

Provisional

Return When Serial Number Is Assigned

Please return this card, indicating receipt date and Serial No., if applicable, of the following

Applicant(s): Bert Vogelstein et al

Title: Converting Diploidy to Haploidy for Genetic Diagnosis

Filing Date: 10/8/99

Client: JHU

BBM&B No.: 01107.8245F

Client Reference: JHU-3574

Attorney/Secretary: SAK/amg

Serial No.: TBA

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Patent/Design

☐ PATENT ☐ DESIGN

☐ HAND CARRY Group/Section _____ Bldg _____ Rm _____

Serial No. TBA B&W # 01107.82458 Atty/Sec SAK/amg Date 10/8/99

Inventor Bert Vogelstein et al Client JHU

Title Converting Diploidy to Haploidy for Genetic Diagnosis

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

X 22 total pp Spec., including : # of Claims 48 (# of independent claims _____); ~~☒ Abstract~~

☐ Foreign Priority on _____ Country, Appl. # and Date

☐ Priority on U.S. Prov. _____ B&W# _____

☐ Application : ☐ CIP ☐ Continuation ☐ Divisional Parent Ser. No. _____ B&W# _____

☐ Provisional App ____ pp Spec/Clms; Cover Sheet

☐ Declaration/PoA : ☐ Executed ☐ Unexecuted

X Drawings : ☐ Formal ☐ Informal # of distinct sheets 4 : Figs. 1-4

☐ Assignment w/PTO Cover Sheet

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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c)(2)

Docket Number	01107.82458	Type a plus sign (+) inside this box →	+

INVENTOR(s)/APPLICANT(s)			
LAST NAME	FIRST NAME	M.I.	RESIDENCE (CITY and either STATE or COUNTRY)
VOGELSTEIN KINZLER	Bert Kenneth	 W.	Baltimore, Maryland Belair, Maryland

TITLE OF THE INVENTION (280 characters max)

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

CORRESPONDENCE ADDRESS

BANNER & WITCOFF, LTD.
Eleventh Floor
1001 G Street, N.W.

STATE Washington, D.C.	ZIP 20001-4597	COUNTRY USA

ENCLOSED APPLICATION PARTS *(check all that apply)*

X	Specification	Number of Pages	22		Small Entity Statement	
X	Drawings	Number of Sheets	4.00	X	Other *(specify)*	CLAIMS 48

METHOD OF PAYMENT *(check one)*

	A check or money order is enclosed to cover the Provisional filing fee			
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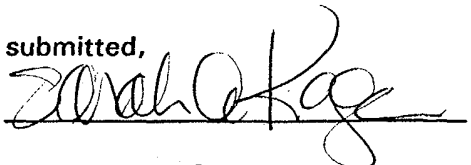
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

___ NO

_X_ YES, the name of the U.S. Government agency and the Government contract number are: National Institute of Health CA57345, CA62924, CA43460, CA67409, and CA72851

Respectfully submitted,

SIGNATURE  DATE October 8, 1999

TYPED or PRINTED NAME Sarah A. Kagan REG. NO. *(if appropriate)* 32,141

___ Additional inventors are being named on separately numbered sheets attached hereto

PROVISIONAL APPLICATION FILING ONLY

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CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

This invention was supported with U.S. government funds, NIH grants CA43460, CA57345, CA62924, CA67409, CA72851. The government therefore retains certain rights in the invention.

BACKGROUND OF THE INVENTION

The problem with humans, at least from a genetic diagnostic perspective, is that they are diploid. Mutations in one allele, such as those responsible for all dominantly inherited syndromes, are always accompanied by the wild-type sequence of the second allele. Though many powerful techniques for genetic diagnosis have been developed over the past decade, all are compromised by the presence of diploidy in the template. For example, the presence of a wild-type band of the same electrophoretic mobility as a mutant band can complicate interpretation of sequencing ladders, especially when the mutant band is of lower intensity. Deletions of a segment of DNA are even more problematic, as in such cases only the wild-type allele is amplified and analyzed by standard techniques. These issues present difficulties for the diagnosis of monogenic diseases and are even more problematic for multigenic diseases, where causative mutations can occur in any of several different genes. Such multigenism is the rule rather than the exception for common predisposition syndromes, such as those associated with breast and colon cancer, blindness, and hematologic, neurological, and cardiovascular diseases. The sensitivity of genetic diagnostics for these diseases is currently suboptimal, with 30% to 70% of cases refractory to genetic analysis.

There is a need in the art for simply separating and analyzing individual alleles from human cells.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for detecting mutations in a gene of interest on a human chromosome.

It is another object of the invention to provide a method for making test cells suitable for sensitive genetic testing.

It is yet another object of the invention to provide a population of fused cell hybrids which are useful for genetic analysis.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method of detecting mutations in a gene of interest of a human is provided. Cells of a human are fused to rodent cell recipients to form human-rodent cell hybrids. Fused cell hybrids are selected by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human chromosome, forming a population of fused cell hybrids. A subset of hybrids are detected among the population of fused cell hybrids. The hybrids are haploid for a second human chromosome which is not the same chromosome as the first human chromosome and which was not selected. The subset of hybrids are tested to detect a gene, an mRNA product of said gene, or a protein product of said gene. The gene resides on the second human chromosome. Diminished amounts of the mRNA or protein product or altered properties of the gene, mRNA, or protein product indicate the presence of a mutation in the gene in the human.

According to another embodiment, a method is disclosed which provides test cells for genetic testing. The test cells are haploid for human genes. Cells of a human are fused to transformed, diploid, rodent cell recipients to form human-rodent cell hybrids. Fused cell hybrids are selected by selecting for a marker on each of a first human chromosome and a rodent chromosome, forming a population of cells which stably maintain one or more human chromosomes in the absence of selection for the human chromosomes. Cells which are haploid for a second human chromosome which is distinct from the first human chromosome are detected among the population of cells; the second human chromosome was not selected.

Also provided by the present invention is a population of rodent-human hybrid cells wherein each homolog of at least 2 human autosomes is present in haploid form in at least one out of one hundred of the cells.

The present invention thus provides the art with a method which can be used to increase the sensitivity and effectiveness of various diagnostic and analytic methods by providing cells to analyze which are haploid for one or more genes of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Strategy for hybrid generation. The recipient mouse cell line E2 was fused with human lymphocytes and clones were subsequently selected with HAT plus geneticin, which kill unfused E2 cells and lymphocytes, respectively. All clones contained a human X chromosome responsible for growth in HAT. Clones were genotyped to determine which human chromosomes were retained. Chromosomes marked "A" and "B" represent the two homologs of a representative human chromosome. The average proportion of clones which retained neither, both, or either of the six chromosome homologs analyzed is indicated (see text). Mutational analysis was carried out on nucleic acids of clones which retained single alleles of the genes to be tested.

Fig. 2. Allelic status and gene expression in hybrids. **(Fig. 2A)** Polymorphic markers from the indicated chromosomes were used to determine the genotype of the indicated hybrids. "Donor" denotes the human lymphocytes used for fusion with the mouse recipient cells. **(Fig. 2B)** cDNA of E2 and four hybrids were used as templates to amplify *hMSH2*, *hMLH1*, *hTGF β-RII*, *hPMS1*, *hPMS2*, and *APC* sequences. The results were concordant with the genotypes observed in **(Fig. 2A)**, in that hybrids 5 - 7 retained at least one allele of each of the chromosomes containing the tested genes, while hybrid 8 contained alleles of chromosomes 3, 5, and 7 but not of chromosome 2 (containing the *hMSH2, hPMS1, and hMSH6* genes).

Fig. 3. Mutational analysis of an HNPCC patient refractory to standard genetic diagnosis. Nucleic acids from the indicated hybrids were tested for retention of chromosomes 2 and 3 using polymorphic markers (**Fig. 3A**) and for expression of *hMSH2* and *hMLH1* genes on chromosomes 2 and 3, respectively (**Fig. 3B**). Hybrids 1, 2, 3, and 6 contained allele A from chromosome 2 and did not express hMSH2 transcripts, while hybrids 4 and 5 contained the B allele and expressed hMSH2. hMLH1 expression served as a control for the integrity of the cDNA. (**Fig. 3C**) Sequences representing the indicated exons of *hMSH2* were amplified from the indicated hybrids. Exons 1- 6 were not present in the hybrids containing allele A, but exons 7 - 16 were present in hybrids containing either allele.

Fig. 4. Mutational analysis of Warthin family G. (**Fig. 4A**) Sequence analysis of RT-PCR products from hMSH2 transcripts of hybrid 1, containing the mutant allele of a Warthin family G patient, illustrates a 24 bp insertion (underlined; antisense primer used for sequencing). The wild-type sequence was found in hybrid 3, containing the wt allele. RT-PCR analysis of transcripts from lymphoid cells of the patient showed that the mutant transcript was expressed at significantly lower levels than the wild-type sequence. Sequence analysis of the genomic DNA of the same hybrids (**Fig. 4B**) showed that the insertion was due to a A to C mutation (antisense sequence, indicated in bold and underlined) at the splice acceptor site of exon 4, resulting in the use of a cryptic splice site 24 bp upstream. The signal of the mutant C is not as strong as the wild-type A in the donor's DNA. Such non-equivalence is not unusual in sequencing templates from diploid cells, and can result in difficulties in interpretation of the chromatograms. (**Fig. 4C**) Extracts from hybrids 1 and 5, carrying the mutant allele of chromosome 2, were devoid of hMSH2 protein, while extracts of hybrids 2 and 3, carrying the wt allele, contained hMSH2 protein. Hybrid 4 did not contain either allele of chromosome 2. Hybrids 1, 3, 4, and 5 each carried at least one allele of chromosome 3 and all synthesized hMLH1 protein. α-tubulin served as a protein loading control. Immunoblots with antibodies to the indicated proteins are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have devised a strategy for generating hybrids containing any desired human chromosome using a single fusion and selection condition. Importantly and unexpectedly, the human chromosomes in these hybrids were stable, and they expressed human genes at levels sufficient for detailed analysis. The approach is based on the principle that fusion between human and rodent cells creates hybrid cells that contain the full rodent genomic complement but only a portion of the human chromosomes. In the past, selection for retention of a specific human chromosome (by complementation of an auxotrophic rodent cell, for example) has allowed the isolation of hybrids containing a desired chromosome (7, 8). Though such fusions have proven useful for a variety of purposes (8, 9), their utility has been limited by the availability of appropriate rodent recipients for many chromosomes and by the inefficiencies and variation of the fusion and selection conditions. For the analysis of multigenic diseases, it would be necessary to perform a separate fusion and selection for each chromosome.

The stability of the human chromosomes in the hybrids of the present invention was surprising. Though the human genetic constitution of radiation hybrids is relatively stable, this stability has been presumed to be due to the integration of small pieces of human DNA into rodent chromosomes following irradiation of the donor cells. The human chromosomes in whole cell fusions have been believed to be unstable unless continuous selection pressure for individual chromosomes was exerted. The reasons for the stability in our experiments is unclear, but may be related to the diploid nature of the rodent partner. Such diploidy reflects a chromosome stability that is unusual among transformed rodent cells. Previous experiments have indeed shown that chromosomally stable human cells retain all chromosomes upon fusion with other chromosomally stable human cells, unlike the situation when one of the two partners is chromosomally unstable.

The diploid, rodent recipient cells of the present invention provide useful reagents for the facile creation of cells with functionally haploid human genomes. Nucleic acids or proteins from these hybrids can be used as reagents for any standard mutational assay. As mutational assays are constantly being improved and automated (1), the value of the hybrid-generated materials correspondingly increases. It may

soon become possible, in fact, to examine the sequence of entire genes (promoters and introns in addition to exons). Nucleic acid templates generated from single alleles are clearly superior for such analyses, as the homogeneous nature of the templates dramatically enhances the signal to noise ratio of virtually any diagnostic assay. We therefore envision that this approach can be productively applied to a wide variety of research and clinical problems.

Genes of interest are typically those which have been found to be involved in inherited diseases. These include genes involved in colon cancer, breast cancer, Li-Fraumeni disease, cystic fibrosis, neurofibromatosis type 2, von Hippel-Lindau disease, as well as others. The identified genes include *APC*, *merlin*, *CF*, *VHL*, *hMSH2*, *p53*, *hPMS2*, *hMLH1*, *BRAC1*, as well as others. Mutations which can be identified at the protein level include those in sequences that regulate transcription or translation, nonsense mutations, splice site alterations, translocations, deletions, and insertions, or any other changes that result in substantial reduction of the full-length protein. Other subtler mutations can be detected at the nucleic acid level, such as by sequencing of RT-PCR products.

Cells of the human which may be used in fusions are any which can be readily fused to rodent cells. Peripheral blood lymphocytes (PBL) which are readily available clinical specimens are good fusion partners, with or without prior mitogenetic stimulation, whether used fresh or stored for over one year at -80° C. Since inherited mutations are the subject of the present method, any cells of the human body can be used, since all such cells contain essentially the same genetic complement.

Rodent cell recipients for fusion are preferably diploid, more preferably oncogene-transformed, and even more preferably have microsatellite instability due to a defect in a mismatch repair gene. Selection of particular clones which grow robustly, are stably diploid, and fuse at a high rate is well within the skill of the ordinary artisan.

Fusion of cells according to the present invention can be accomplished according to any means known in the art. Known techniques for inducing fusion include polyethylene glycol-mediated fusion, Sendai virus-mediated fusion, and electro-fusion. Cells can desirably be mixed at a ratio of between 10:1 and 1:10

human to rodent. Clones of fused cells generally become visible after about two to three weeks of growth.

Fused hybrid cells can be selected using any markers which result in a positively selectable phenotype. These include antibiotic resistance genes, toxic metabolite resistance genes, protrophic markers, etc. The surprising advantage of the present invention is that a single marker on a single human chromosome can be used in the selection, and that stable hybrids containing more than just the single, selected human chromosome result. Thus markers on other chromosomes can be analyzed even when the chromosomes on which the markers reside were not selected.

Fused hybrid cells can be analyzed to determine that they do in fact carry a human chromosome which carries a gene of interest. Hybrid cells which have either of the two relevant human chromosomes can be distinguished from each other as well as from hybrids which contain both of the two human chromosomes. See **Fig. 1.** While any means known in the art for identifying the human chromosomes can be used, a facile analysis can be performed by assessing microsatellite markers on the human chromosome. Other linked polymorphic markers can be used to identify a desired human chromosome in the hybrids.

Once hybrid cells are isolated which contain one copy of a human gene of interest from a human who is being tested, mutation analysis can be performed on the hybrid cells. Mutations that result in reduced expression of the full-length gene product should be detectable by Western blotting using appropriate antibodies. Tests which rely on the function of the protein encoded by the gene of interest and enzyme assays can also be performed to detect mutations. Other immunological techniques can also be employed, as are known in the art.

If an immunological method is used to detect the protein product of the gene of interest in the hybrids, it is desirable that antibodies be used that do not cross-react with rodent proteins. Alternatively, the rodent genes which are homologous to the gene of interest can be inactivated by mutation to simplify the analysis of protein products. Such mutations can be achieved by targeted mutagenesis methods, as is well known in the art.

Functional tests can also be used to assess the normalcy of each allelic product. For example, if one inserted an expression construct comprising a β-galactosidase

gene downstream from a p53 transcriptional activation site, into a rodent-human hybrid cell that contained human chromosome 17 but no endogenous p53, then one could detect mutations of the p53 on the human chromosome 17 by staining clones with X-gal. Other enzymatic or functional assays can be designed specifically tailored to the gene of interest.

Any method of detecting mutations at the DNA or RNA level as are known in the art may be employed. These include without limitation, sequencing, allele-specific PCR, allele-specific hybridization, microarrays, DGGE, and automated sequencing.

It is a possibility that expression of the gene of interest might be inhibited in the hybrid cell environment. In order for the loss of expression of a gene of interest in the hybrid cells to be meaningfully interpreted as indicating a mutation in the human, one must confirm that the gene of interest, when wild-type, is expressed in rodent-human hybrid cells. This confirmation need not be done for each patient, but can be done once when the assay is being established.

When the assay of the present invention indicates that a mutation exists in the gene of interest, other family members can be tested to ascertain whether they too carry the mutation. Alternatively, the other family members can be tested to see if they carry the same chromosome as the affected family member. This can be determined by testing for a haplotype, i.e., a set of distinctive markers which are found on the chromosome carrying the mutation in the affected family member. Determination of a haplotype is a by-product of performing the assay of the invention on the first family member. When the hybrid cells are tested to confirm the presence of the relevant chromosome in the hybrid, for example by use of microsatellite markers, a distinctive marker set will be identified, which can then be used as a haplotype.

Populations of hybrid cells made by the fusion process of the present invention may contain hybrid cells which are haploid for a number of different human chromosomes. Typically each homolog of at least 2, at least 5, at least 10, at least 15, at least 20, or even 22 human autosomes will be present in the population in a haploid condition in at least one out of one hundered, seventy-five, fifty, thirty or twenty-eight of the cells. Thus a high proportion of the cells contain multiple human

chromosomes, and a relatively small number of cells must be tested to find cells harboring a single copy of a non-selected chromosome.

The following examples provide experimental details which demonstrate one of many ways to carry out the invention. The invention is not limited to the particular methods of cells employed in the examples. The claims and the specification as a whole provide the measure of the invention.

Examples

Example 1

An outline of the approach is presented in Fig. 1. The rodent fusion partner was a line derived from mouse embryonic fibroblasts transformed with ras and adenovirus E1A oncogenes. HPRT-deficient subclones of this line were generated, and one subclone (E2) was chosen for further experimentation based on its robust growth characteristics, maintenance of diploidy, and fusion efficiency (10). Human lymphocytes cells were mixed with E2 cells at an optimum ratio and electrofused, and hybrids selected in geneticin (to kill unfused human cells) and HAT (to kill unfused E2 cells) (11). Colonies appearing after two weeks of growth were expanded and RNA and DNA prepared for analysis. From a single fusion experiment, an average of 36 hybrid clones were obtained (range of 17 to 80 in five different individuals).

All hybrids contained the human X chromosome, as this chromosome contains the HPRT gene allowing growth in HAT. To determine whether other human chromosomes were present in the hybrids, polymorphic microsatellite markers (12) were used as probes in PCR-based assays (Fig. 2A). We focused on the chromosome arms (2p, 2q, 3p, 5q, 7q, and 16q) known to contain colorectal cancer (CRC) predisposition genes. One copy of each of these chromosome arms was present in a significant fraction of the hybrid clones. For example, of 91 hybrids examined for chromosome 3, 26 hybrids contained neither donor chromosome, 27 hybrids contained both donor chromosomes, 17 hybrids contained the maternal chromosome, and 21 hybrids contained the paternal chromosome. Similar retention frequencies were found for all six chromosome arms analyzed. Testing of markers from both arms of a single chromosome showed that whole chromosomes, rather than chromosome fragments, were generally retained in the hybrids. This result was confirmed with fluorescence in situ hybridization (FISH) on metaphase spreads from the hybrids (data not shown).

Calculations based on the genotypic data indicated that the analysis of 15 hybrids would ensure a 95% probability of identifying at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of a single chromosome under study. Moreover, it would require only 28 hybrids to similarly ensure that each allele of all 22 autosomes was present and separated from its homolog in at least one hybrid (13).

Example 2

Two other features of the hybrids were essential for the analyses described below. First, the human chromosome complements of the hybrids were remarkably stable. Polymorphic marker analysis in ten hybrids revealed identical patterns of retention after growth for 72 generations after initial genotyping. Second, those hybrids containing the relevant chromosome expressed every human gene assessed, including the *hMSH2* and *hMSH6* genes on chromosome 2p, the *hPMS1* gene on chromosome 2q, the *TGF-β Receptor Type II* gene and *hMLH1* gene on chromosome 3p, the *APC* gene on chromosome 5q, the *hPMS2* gene on chromosome 7q, and the *E-cadherin* gene on chromosome 16q (representative examples in Fig. 2B) (14).

Example 3

Having established the stability and expression patterns of CRC-predisposition genes in these hybrids, we used this approach to investigate a classic hereditary non-polyposis colorectal cancer (HNPCC) kindred in which standard mutational analysis had not been informative. This prior analysis included complete sequencing of the cDNA of all five MMR genes known to cause HNPCC. Hybrids were generated from lymphocytes of an affected individual from this kindred, and hybrids containing each allele of the *hMSH2* and *hMLH1* genes were tested for expression using RT-PCR. The *hMLH1* gene was expressed in all hybrids containing chromosome 3. However, hybrids containing allele A of chromosome 2 did not express a full length *hMSH2* transcript, while hybrids containing allele B of chromosome 2 did (Fig. 3A, B). Further experiments showed that exons 1 - 6 were completely missing in the hybrids containing allele A, which still contained exons 7 - 16 (Fig. 3C). The absence of these exons explained why no mutations were detected in RT-PCR analyses of the patient's lymphocytes. The deletion of these exons also

explained why no mutation was detectable upon analysis of individual exons in the genomic DNA from the lymphocytes; only the wild-type allele would be PCR-amplified in such assays.

Example 4

We next evaluated Warthin Family G, a kindred which was described in 1895 as the first example of a hereditary colorectal cancer syndrome. Like the kindred described above, standard mutational analyses of the transcripts from every MMR gene had failed to reveal a mutational basis for the disease in this family. Analysis of appropriate hybrids showed that all five MMR genes were expressed as RT-PCR products in hybrids containing the relevant loci. We therefore used the RT-PCR products as templates for automated sequencing (18). This analysis revealed an aberrant *hMSH2* sequence in one hybrid, and a wild-type sequence in the hybrid containing the other allele (Fig. 4A). The aberrant sequence consisted of a 24 bp insertion predicted to result in an in-frame addition of eight amino acids between codons 215 and 216. Sequencing of the relevant region of the *hMSH2* gene from these hybrids revealed a T to G transversion residue three bases upstream of exon 4 (Fig. 4B). This abrogated the normal splice site, revealing a cryptic splice site 24 bp upstream. Direct sequence analysis of RT-PCR products from the patient's lymphocytes showed that the mutant allele was expressed at levels considerably lower than that from the wild-type allele, thus explaining why it had not been detected in standard sequencing analyses of lymphocyte cDNA (Fig. 4A). To demonstrate that this mutation had an effect at the protein level, we analyzed the hybrids by immunoblotting with specific antibodies (19). The hybrids containing the mutant allele did not make detectable levels of human hMSH2 protein, though they did synthesize normal levels of a control protein (Fig. 4C).

The results described above demonstrate that individual alleles of human chromosomes can be readily isolated upon fusion to mouse cells.

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9. H. Harris, *J Cell Sci* **79**, 83 (1985); M.J. Anderson, E.J. Stanbridge, *FASEB J* **7**, 826(1993).

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[1]All references are explicitly incorporated by reference.

10. E2 cells were derived from mouse embryonic fibroblasts derived from MSH2-deficient mice (generously provided by T. Mak) and transformed with adenovirus *E1A* and *RAS* oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

11. 3×10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX Electro Cell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersberg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. The lymphocytes used for the experiments described here were derived from Epstein-Barr virus infection of peripheral blood leukocytes, but we found that freshly drawn lymphocytes could also be successfuly fused and analyzed using identical methods

12. Genotyping was performed as described in F.S. Leach et al., *Cell* 75, 1215 (1993). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788, D2S13360, D3S2406, D7S1824, and D16S3095, from chromosomes 2p, 2q, 3p, 5q, and 16q, respectively.

13. The numbers of hybrids containing none, both, or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the numbers of hybrids required to generate mono-allelic hybrids containing specific numbers of chromosomes.

14. Polyadenylated RNA was purified and RT-PCR performed as described in B. Liu et al., *Nat Medicine* **2**, 169 (1996).

15. C.R. Boland, *Am. J. Dig. Dis* **23**, 25s-27s (1978); C.R. Boland, West *J. Med.* **139**, 351 (183).

16. C.R. Boland, in *The Genetic Basis of Human Cancer,* B. Vogelstein and K.W. Kinzler, Eds (McGraw-Hill, New York, 1998), pp. 333-346.

17. A.S. Warthin, *Archives of Internal Medicine* **12**, 546 (1913; H.T. Lynch and A.J. Krush, *Cancer* **27**, 1505 (1971).

18. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through a *Science* internet site.

19. Cytoplasmic extracts of cells hybrids were separated by electrophoresis through SDS-polyacrylamide gels and immunoblotted with antibodies specific for human hMSH2 (#NA26, Calboiochem), human hMLH1 (#13271A, Pharmingen), or β-Tubulin (#N357, Amersham).

20. D.R. Cox, *Cytogenet Cell Genet* **59**, 80 (1992); M.A. Walter, D.J. Spillett, P. Thomas, J. Weissenbach, P.N. Goodfellow, Nat Genet **7**, 22 (1994); R.J. Leach, P. O'Connell, *Adv Genet* 33, 63 (1995).

21. C. Lengauer, K.W. Kinzler, B. Vogelstein, *Nature* **386**, 623 (1997).

22. M. Chee et al., *Science* 274, 610 (1996).

CLAIMS

1. A method of detecting mutations in a gene of interest of a human, comprising the steps of:

fusing cells of a human to rodent cell recipients to form human-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human chromosome, to form a population of fused cell hybrids;

detecting among the population of fused cell hybrids a subset of hybrids which are haploid for a second human chromosome which is not the same chromosome as the first human chromosome and which was not selected;

testing said subset of hybrids to detect a gene, an mRNA product of said gene, or a protein product of said gene, wherein the gene resides on the second human chromosome, wherein diminished amounts of said mRNA or protein product or altered properties of said gene, mRNA, or protein product indicate the presence of a mutation in the gene in the human.

2. The method of claim 1 wherein the rodent cell is diploid.

3. The method of claim 1 wherein the rodent cell recipients are deficient in mismatch repair.

4. The method of claim 1 wherein the rodent cell recipient is geneticin resistant.

5. The method of claim 1 wherein the rodent cell recipients are transformed with an oncogene.

6. The method of claim 5 wherein the oncogene is *ras*.

7. The method of claim 5 wherein the oncogene is *E1A*.

8. The method of claim 1 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

9. The method of claim 1 wherein the rodent cell recipients are *hMSH2*$^-$.

10. The method of claim 1 wherein the rodent cell recipient is diploid, and contains both a selectable marker and a counterselectable marker.

11. The method of claim 10 wherein the counterselectable marker is HPRT deficiency.

12. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third human chromosome, wherein the first, second, and third human chromosomes are distinct, and neither the second nor the third human chromsomes were selected.

13. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third, fourth, fifth, and sixth human chromosome, wherein the first through sixth human chromosomes are distinct, and neither the second through sixth human chromsomes were selected.

14. The method of claim 1 wherein the cells of the human are lymphocytes.

15. The method of claim 1 wherein the step of detecting a subset of hybrids which are haploide for the second human chromosome is accomplished by identifying the presence of a microsatellite marker on the second human chromosome.

16. A method of providing test cells for genetic testing, wherein said test cells are haploid for human genes, comprising the steps of:

fusing cells of a human to transformed, diploid, rodent cell recipients to form human-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a marker on each of a first human chromosome and a rodent chromosome, whereby a population of cells are formed which stably maintain one or more human chromosomes in the absence of selection for the human chromosomes;

detecting among the population of cells those cells which are haploid for a second human chromosome which is distinct from the first human chromosome and which was not selected.

17. The method of claim 16 wherein the rodent cell recipient is geneticin resistant.

18. The method of claim 16 wherein the rodent cell recipients are transformed with an oncogene.

19. The method of claim 18 wherein the oncogene is *ras*.

20. The method of claim 18 wherein the oncogene is *E1A*.

21. The method of claim 16 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

22. The method of claim 16 wherein the rodent cell recipients are deficient in mismatch repair.

23. The method of claim 16 wherein the rodent cell recipient contains a counterselectable marker.

24. The method of claim 23 wherein the counterselectable marker is HPRT deficiency.

25. The method of claim 16 wherein the cells of the human are lymphocytes.

26. The method of claim 16 wherein the step of detecting cells which are haploid for said second human chromosome is performed by identifying the presence of a microsatellite marker on said second human chromosome.

27. The method of claim 16 further comprising the step of:

detecting among the population of cells, cells haploid for a third human chromosome which is distinct from the first and second human chromosomes and which was not selected.

28. The method of claim 16 futher comprising the step of:

detecting among the population of cells, cells haploid for a third, fourth, fifth, and sixth human chromosome, wherein said third through sixth human chromosomes are distinct from the first and second human chromosomes and were not selected.

29. The method of claim 16 further comprising:

testing nucleic acids of a cell haploid for the second human chromosome for a mutation in a gene on the second human chromosome.

30. The method of claim 16 further comprising:

testing proteins of a cell haploid for the second human chromosome for a mutation in a gene on the second human chromosome.

31. The method of claim 1 wherein nucleic acids of a cell in the subset of hybrids are tested for a mutation in a gene on the second human chromosome.

32. The method of claim 1 wherein proteins of a cell in the subset of hybrids are tested for a mutation in a gene on the second human chromosome.

33. A population of rodent-human hybrid cells wherein each homolog of at least 2 human autosomes is present in haploid form in at least one out of one hundred of the cells.

34. The population of claim 33 wherein each homolog of at least 5 human autosomes is present in haploid form in at least one out of a hundred of the cells.

35. The population of claim 33 wherein each homolog of at least 5 human autosomes is present in haploid form in at least one out of fifty of the cells.

36. The population of claim 33 wherein each homolog of at least 5 human autosomes is present in haploid form in at least one out of thirty of the cells.

37. The population of claim 33 wherein each homolog of at least 10 human autosomes is present in haploid form in at least one out of a hundred of the cells.

38. The population of claim 33 wherein each homolog of at least 10 human autosomes is present in haploid form in at least one out of fifty of the cells.

39. The population of claim 33 wherein each homolog of at least 10 human autosomes is present in haploid form in at least one out of a thirty of the cells.

40. The population of claim 33 wherein each homolog of at least 15 human autosomes is present in haploid form in at least one out of a hundred of the cells.

41. The population of claim 33 wherein each homolog of at least 15 human autosomes is present in haploid form in at least one out of fifty of the cells.

42. The population of claim 33 wherein each homolog of at least 15 human autosomes is present in haploid form in at least one out of thirty of the cells.

43. The population of claim 33 wherein each homolog of at least 20 human autosomes is present in haploid form in at least one out of a hundred of the cells.

44. The population of claim 33 wherein each homolog of at least 20 human autosomes is present in haploid form in at least one out of fifty of the cells.

45. The population of claim 33 wherein each homolog of at least 20 human autosomes is present in haploid form in at least one out of thirty of the cells.

46. The population of claim 33 wherein each homolog of at least 22 human autosomes is present in haploid form in at least one out of a hundred of the cells.

47. The population of claim 33 wherein each homolog of at least 22 human autosomes is present in haploid form in at least one out of fifty of the cells.

48. The population of claim 33 wherein each homolog of at least 22 human autosomes is present in haploid form in at least one out of thirty of the cells.

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

ABSTRACT OF THE DISCLOSURE

Detection of mutations associated with hereditary diseases is complicated by the diploid nature of human cells. Mutations present in one allele are often masked by the wild-type sequence of the other allele. Individual alleles can be isolated from every chromosome within somatic cell hybrids generated from a single fusion. Nucleic acids from the hybrids can be analyzed for mutations in an unambiguous manner. This approach was used to detect two cancer-causing mutations that had previously defied genetic diagnosis. One of the families studied, Warthin Family G, was the first kindred with a hereditary colon cancer syndrome described in the biomedical literature.

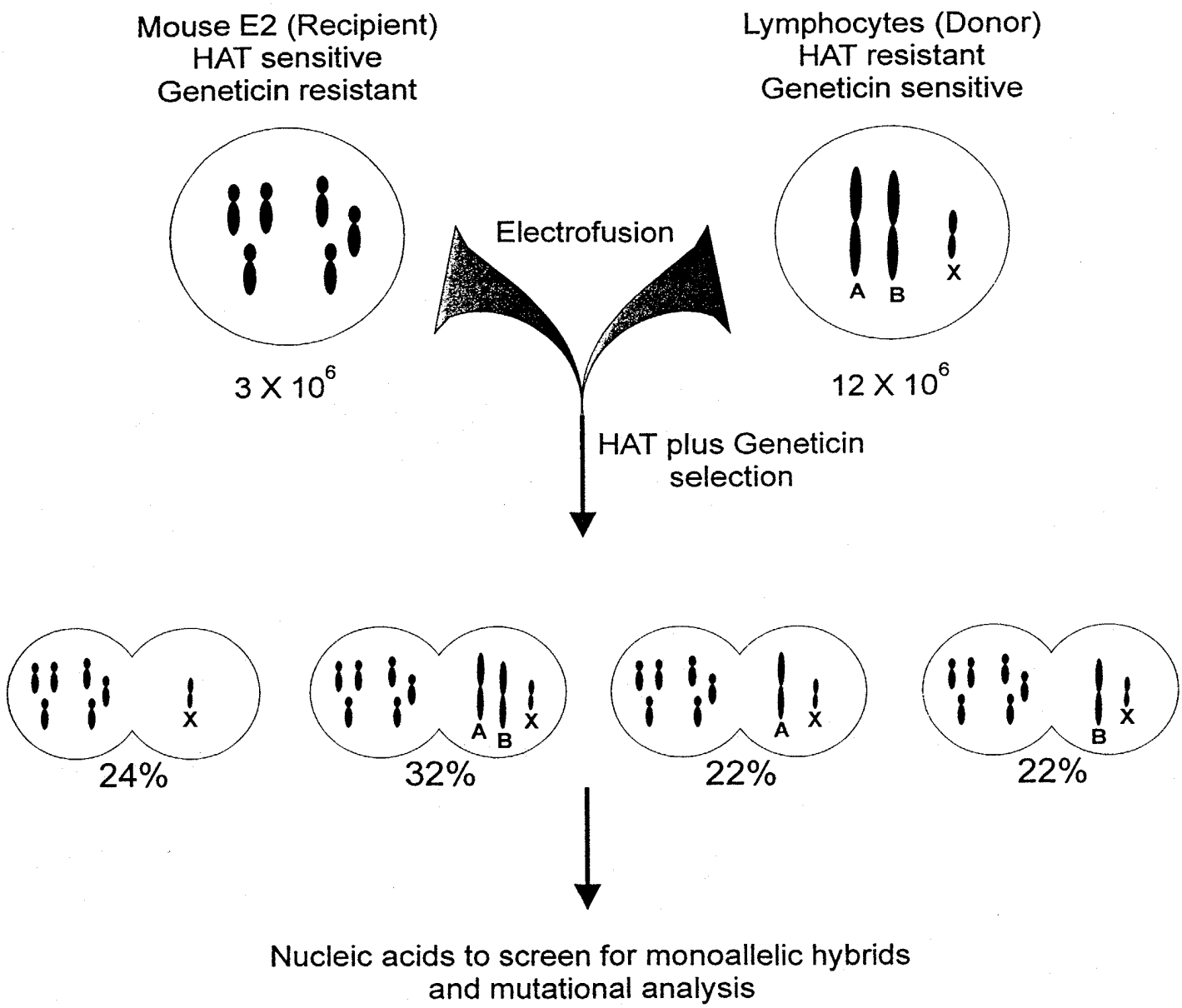

Figure 1
Yan *et al.*

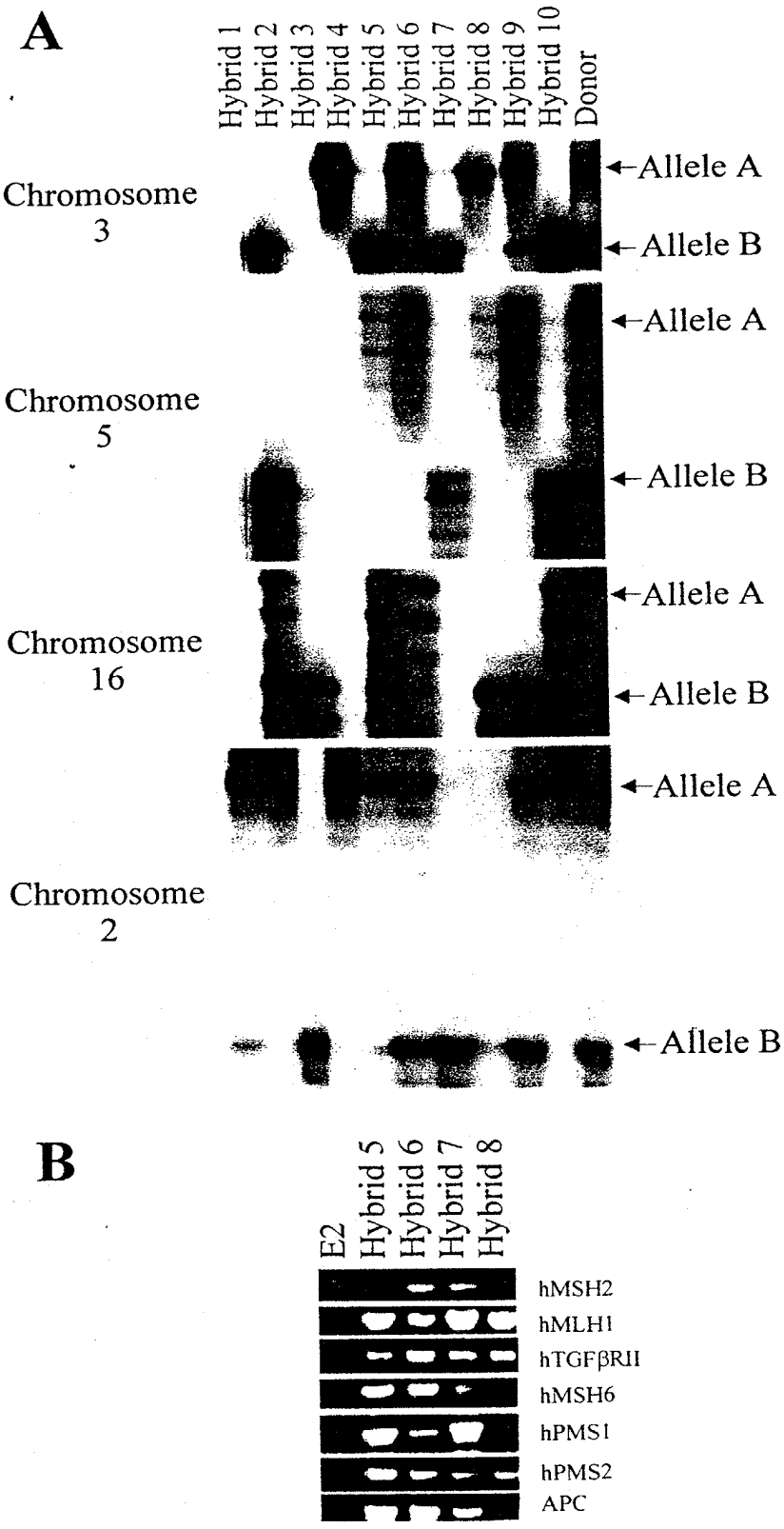

Figure 2
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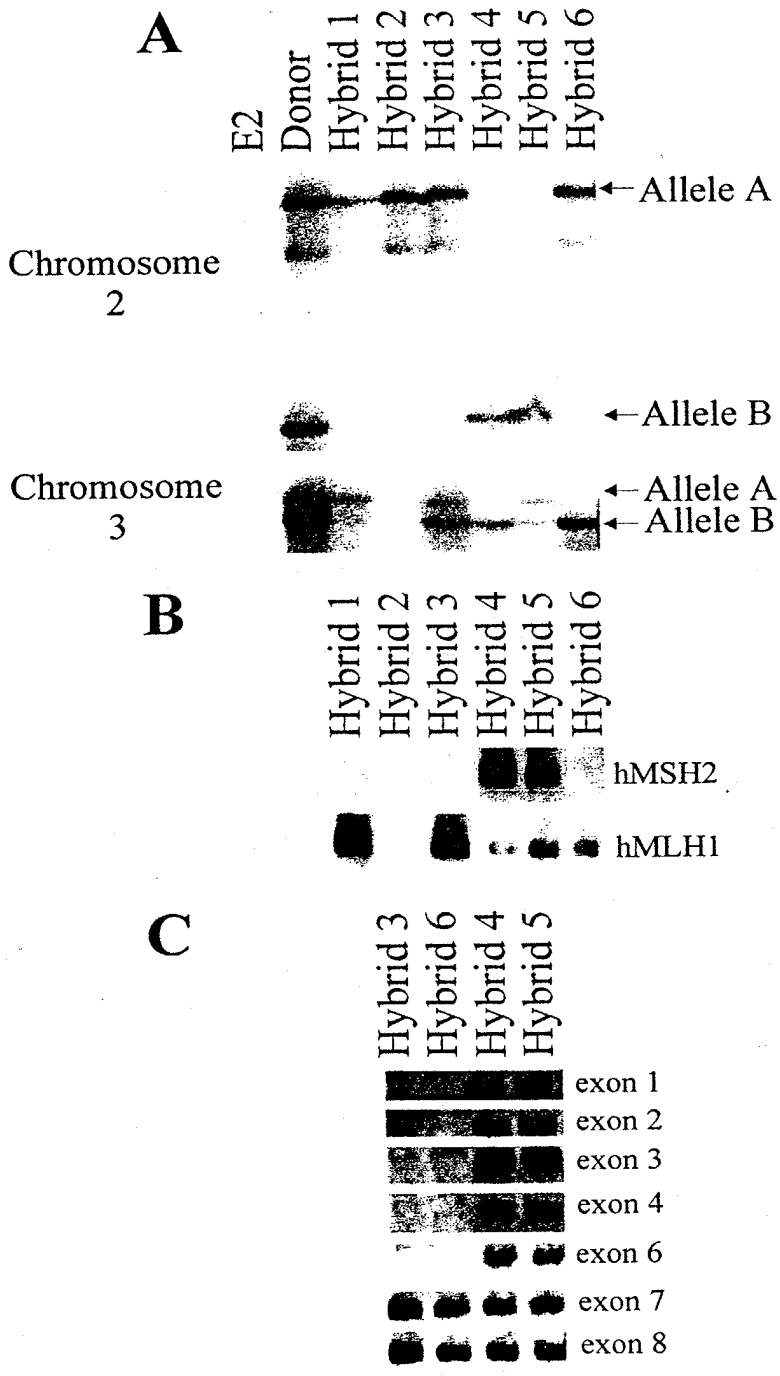

Figure 3
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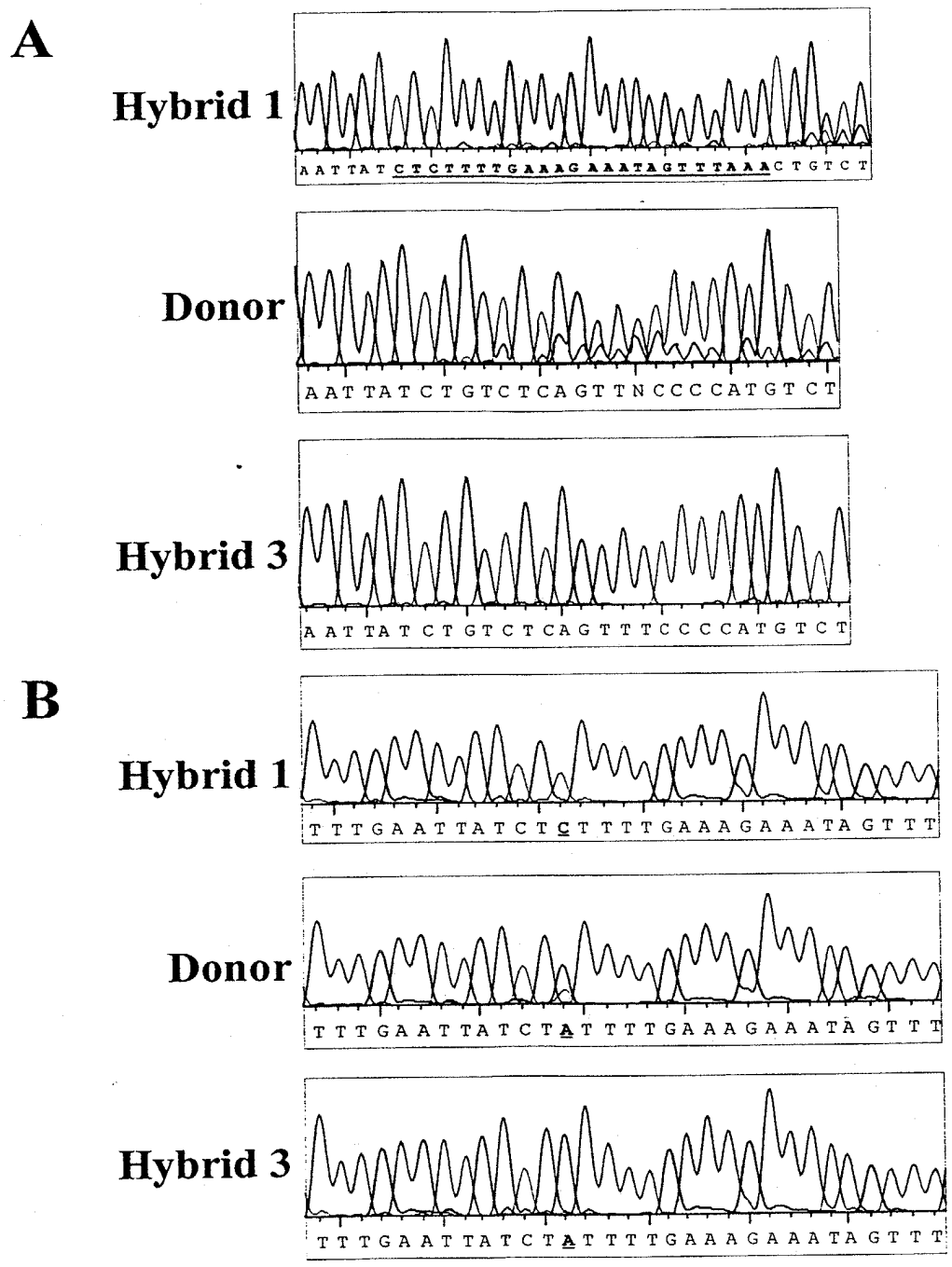

Figure 4
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09/461,047	12/15/1999	1643	781	01107.84957	4	53	4

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Applicant(s)

BERT VOGELSTEIN, Baltimore, MD ;
Kenneth W. Kinzler, Bel Air, MD ;
Hai Yan, Baltimore, MD ;
Nicholas Papadopoulos, New York, NY ;

Continuing Data as Claimed by Applicant

THIS APPLN CLAIMS BENEFIT OF 60/158,160 10/08/1999

Foreign Applications

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**** SMALL ENTITY ****

Title

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

Preliminary Class

435

Patent/Design

☐ PATENT ☐ DESIGN

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Serial No. TBA B&W # 011078.4952 Atty/Sec SAK/amq Date 12/15/99

Inventor Vogelstein Client SHG

Title Converting Diploidy to Haploidy for Genetic Diagnosis

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

X 23 total pp Spec., including : # of Claims 53
(# of independent claims 3); X Abstract

☐ Foreign Priority on _____
Country, Appl. # and Date

☐ Priority on U.S. Prov. _____ B&W# _____

☐ Application : ☐ CIP ☐ Continuation ☐ Divisional
Parent Ser. No. _____ B&W# _____

X Provisional App ____ pp Spec/Clms; Cover Sheet

☐ Declaration/PoA : ☐ Executed ☐ Unexecuted

☐ Drawings : ☐ Formal ☐ Informal
# of distinct sheets _____ : Figs. _____

☐ Assignment w/PTO Cover Sheet

☐ Small Entity Declaration

☐ IDS w/PTO 1449 ☐ Prior Art ☐ w/fee

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Applicant(s): Vogelstein

Title: Converting Diploidy to Haploidy for Genetic Diagnosis

JC688 U.S. PTO
09/461047
12/15/99

Filing Date: 12/15/99

Client: JHU

BBM&B No.: 01107.84957 Client Reference:

Attorney/Secretary: SAK/ama

Serial No.: TBA

BBMB-Rev. 8/91 NoFFdKted

NEW UNITED STATES UTILITY PATENT APPLICATION
under 37 C.F.R. 1.53(b)

Atty. Docket No. 01107.84957

Assistant Commissioner of Patents
Box Patent Applications
Washington, D.C. 20231

Enclosed herewith is a new patent application and the following papers:

First Named Inventor (or application identifier): Bert Vogelstein

Title of Invention: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

1. ■ Specification 25 pages (including specification, claims, abstract) / 53 claims (3 independent)

2. ■ Declaration/Power of Attorney is:
   - ☐ attached in the regular manner.
   - ■ NOT included, but deferred under 37 C.F.R. § 1.53(f).

3. ■ 4 Distinct sheets of ■ Formal ☐ Informal Drawings

4. ☐ Preliminary Amendment.

5. ☐ Information Disclosure Statement
   - ☐ Form 1449
   - ☐ A copy of each cited prior art reference

6. ☐ Assignment with Cover Sheet.

7. ■ Priority is hereby claimed under 35 U.S.C. § 119 based upon the following application(s):

Country	Application Number	Date of Filing (day, month, year)
U.S.	60/158,160	08 October 1999

8. ☐ Priority document(s).

9. ☐ Statement Claiming Small Entity Status.

10. ☐ Microfiche Computer Program (Appendix).

11. ☐ Nucleotide and/or Amino Acid Sequence Submission.
   - ☐ Computer Readable Copy.
   - ☐ Paper Copy (identical to computer copy).
   - ☐ Statement verifying identity of above copies.

NEW UNITED STATES UTILITY PATENT APPLICATION
under 37 C.F.R. 1.53(b)

12. Calculation of Fees:

FEES FOR	EXCESS CLAIMS	FEE	AMOUNT DUE
Basic Filing Fee (37 C.F.R. § 1.16(a))			$760.00
Total Claims in Excess of 20 (37 C.F.R. § 1.16(c))	33	18.00	$360.00
Independent Claims in Excess of 3 (37 C.F.R. § 1.16(b))	0	78.00	$0.00
Multiple Dependent Claims (37 C.F.R. § 1.16(d))	0	260.00	$0.00
Subtotal - Filing Fee Due			$3,954.00
	REDUCE BY (%)	($)	
Reduction by 50%, if Small Entity (37 C.F.R. §§ 1.9, 1.27, 1.28)	0		$1,977.00
TOTAL FILING FEE DUE			$1,977.00
Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h))	0	40.00	$0.00
GRAND TOTAL DUE			**$1,977.00**

13. PAYMENT is:

☐ included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18.

■ not included, but deferred under 37 C.F.R. § 1.53(f).

14. All correspondence for the attached application should be directed to:

Banner & Witcoff, Ltd.
1001 G Street, N.W.
Washington, D. C. 20001-4597
Telephone: (202) 508-9100
Facsimile: (202) 508-9299

15. Other: _____

Date: December 15, 1999

By: _____
Sarah A. Kagan
Reg. No. 32,141

SAK/ama

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

This invention was supported with U.S. government funds, NIH grants CA43460, CA57345, CA62924, CA67409, CA72851. The government therefore retains certain rights in the invention. This application claims the benefit of provisional application Serial No. 60/158,160 filed October 8, 1999.

BACKGROUND OF THE INVENTION

The problem with humans, at least from a genetic diagnostic perspective, is that they are diploid. Mutations in one allele, such as those responsible for all dominantly inherited syndromes, are always accompanied by the wild-type sequence of the second allele. Though many powerful techniques for genetic diagnosis have been developed over the past decade, all are compromised by the presence of diploidy in the template. For example, the presence of a wild-type band of the same electrophoretic mobility as a mutant band can complicate interpretation of sequencing ladders, especially when the mutant band is of lower intensity. Deletions of a segment of DNA are even more problematic, as in such cases only the wild-type allele is amplified and analyzed by standard techniques. These issues present difficulties for the diagnosis of monogenic diseases and are even more problematic for multigenic diseases, where causative mutations can occur in any of several different genes. Such multigenism is the rule rather than the exception for common predisposition syndromes, such as those associated with breast and colon cancer, blindness, and hematologic, neurological, and cardiovascular diseases. The sensitivity of genetic diagnostics for these diseases is currently suboptimal, with 30% to 70% of cases refractory to genetic analysis.

There is a need in the art for simply separating and analyzing individual alleles from human cells.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for detecting mutations in a gene of interest on a human chromosome.

It is another object of the invention to provide a method for making test cells suitable for sensitive genetic testing.

It is yet another object of the invention to provide a population of fused cell hybrids which are useful for genetic analysis.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method of detecting mutations in a gene of interest of a human is provided. Cells of a human are fused to rodent cell recipients to form human-rodent cell hybrids. Fused cell hybrids are selected by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human chromosome, forming a population of fused cell hybrids. A subset of hybrids are detected among the population of fused cell hybrids. The hybrids are haploid for a second human chromosome which is not the same chromosome as the first human chromosome and which was not selected. The subset of hybrids are tested to detect a gene, an mRNA product of said gene, or a protein product of said gene. The gene resides on the second human chromosome. Diminished amounts of the mRNA or protein product or altered properties of the gene, mRNA, or protein product indicate the presence of a mutation in the gene in the human.

According to another embodiment, a method is disclosed which provides test cells for genetic testing. The test cells are haploid for human genes. Cells of a human are fused to transformed, diploid, rodent cell recipients to form human-rodent cell hybrids. Fused cell hybrids are selected by selecting for a marker on each of a first human chromosome and a rodent chromosome, forming a population of cells which stably maintain one or more human chromosomes in the absence of selection for the human chromosomes. Cells which are haploid for a second human chromosome which is distinct from the first human chromosome are detected among the population of cells; the second human chromosome was not selected.

Also provided by the present invention is a population of rodent-human hybrid cells wherein each homolog of at least 2 human autosomes is present in haploid form in at least one out of one hundred of the cells.

The present invention thus provides the art with a method which can be used to increase the sensitivity and effectiveness of various diagnostic and analytic methods by providing hybrid cells to analyze which are haploid for one or more genes of interest. The human chromosome content of the hybrid cells is stable and uniform.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Strategy for hybrid generation. The recipient mouse cell line E2 was fused with human lymphocytes and clones were subsequently selected with HAT plus geneticin, which kill unfused E2 cells and lymphocytes, respectively. All clones contained a human X chromosome responsible for growth in HAT. Clones were genotyped to determine which human chromosomes were retained. Chromosomes marked "A" and "B" represent the two homologs of a representative human chromosome. The average proportion of clones which retained neither, both, or either of the six chromosome homologs analyzed is indicated (see text). Mutational analysis was carried out on nucleic acids of clones which retained single alleles of the genes to be tested.

Fig. 2. Allelic status and gene expression in hybrids. **(Fig. 2A)** Polymorphic markers from the indicated chromosomes were used to determine the genotype of the indicated hybrids. "Donor" denotes the human lymphocytes used for fusion with the mouse recipient cells. **(Fig. 2B)** cDNA of E2 and four hybrids were used as templates to amplify *hMSH2, hMSH6, hMLH1, hTGF β-RII, hPMS1, hPMS2,* and *APC* sequences. The results were concordant with the genotypes observed in **(Fig. 2A)**, in that hybrids 5 - 7 retained at least one allele of each of the chromosomes containing the tested genes, while hybrid 8 contained alleles of chromosomes 3, 5, and 7 but not of chromosome 2 (containing the *hMSH2, hPMS1, and hMSH6* genes).

Fig. 3. Mutational analysis of an HNPCC patient refractory to standard genetic diagnosis. Nucleic acids from the indicated hybrids were tested for retention of chromosomes 2 and 3 using polymorphic markers (**Fig. 3A**) and for expression of *hMSH2* and *hMLH1* genes on chromosomes 2 and 3, respectively (**Fig. 3B**). Hybrids 1, 2, 3, and 6 contained allele A from chromosome 2 and did not express hMSH2 transcripts, while hybrids 4 and 5 contained the B allele and expressed hMSH2. hMLH1 expression served as a control for the integrity of the cDNA. (**Fig. 3C**) Sequences representing the indicated exons of *hMSH2* were amplified from the indicated hybrids. Exons 1- 6 were not present in the hybrids containing allele A, but exons 7 - 16 were present in hybrids containing either allele.

Fig. 4. Mutational analysis of Warthin family G. (**Fig. 4A**) Sequence analysis of RT-PCR products from hMSH2 transcripts of hybrid 1, containing the mutant allele of a Warthin family G patient, illustrates a 24 bp insertion (underlined; antisense primer used for sequencing). The wild-type sequence was found in hybrid 3, containing the wt allele. RT-PCR analysis of transcripts from lymphoid cells of the patient showed that the mutant transcript was expressed at significantly lower levels than the wild-type sequence. Sequence analysis of the genomic DNA of the same hybrids (**Fig. 4B**) showed that the insertion was due to a A to C mutation (antisense sequence, indicated in bold and underlined) at the splice acceptor site of exon 4, resulting in the use of a cryptic splice site 24 bp upstream. The signal of the mutant C is not as strong as the wild-type A in the donor's DNA. Such non-equivalence is not unusual in sequencing templates from diploid cells, and can result in difficulties in interpretation of the chromatograms. (**Fig. 4C**) Extracts from hybrids 1 and 5, carrying the mutant allele of chromosome 2, were devoid of hMSH2 protein, while extracts of hybrids 2 and 3, carrying the wt allele, contained hMSH2 protein. Hybrid 4 did not contain either allele of chromosome 2. Hybrids 1, 3, 4, and 5 each carried at least one allele of chromosome 3 and all synthesized hMLH1 protein. α-tubulin served as a protein loading control. Immunoblots with antibodies to the indicated proteins are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have devised a strategy for generating hybrids containing any desired human chromosome using a single fusion and selection condition. Importantly and unexpectedly, the human chromosomes in these hybrids were stable, and they expressed human genes at levels sufficient for detailed analysis. The approach is based on the principle that fusion between human and rodent cells creates hybrid cells that contain the full rodent genomic complement but only a portion of the human chromosomes. In the past, selection for retention of a specific human chromosome (by complementation of an auxotrophic rodent cell, for example) has allowed the isolation of hybrids containing a desired chromosome (7, 8). Though such fusions have proven useful for a variety of purposes (8, 9), their utility has been limited by the availability of appropriate rodent recipients for many chromosomes and by the inefficiencies and variation of the fusion and selection conditions. For the analysis of multigenic diseases, it would be necessary to perform a separate fusion and selection for each chromosome.

The stability of the human chromosomes in the hybrids of the present invention was surprising. Though the human genetic constitution of radiation hybrids is relatively stable, this stability has been presumed to be due to the integration of small pieces of human DNA into rodent chromosomes following irradiation of the donor cells. The human chromosomes in whole cell fusions have been believed to be unstable unless continuous selection pressure for individual chromosomes was exerted. The reasons for the stability in our experiments is unclear, but may be related to the diploid nature of the rodent partner. Such diploidy reflects a chromosome stability that is unusual among transformed rodent cells. Previous experiments have indeed shown that chromosomally stable human cells retain all chromosomes upon fusion with other chromosomally stable human cells, unlike the situation when one of the two partners is chromosomally unstable.

The diploid, rodent recipient cells of the present invention provide useful reagents for the facile creation of cells with functionally haploid human genomes. Nucleic acids or proteins from these hybrids can be used as reagents for any standard mutational assay. As mutational assays are constantly being improved and automated (1), the value of the hybrid-generated materials correspondingly increases. It may

soon become possible, in fact, to examine the sequence of entire genes (promoters and introns in addition to exons). Nucleic acid templates generated from single alleles are clearly superior for such analyses, as the homogeneous nature of the templates dramatically enhances the signal to noise ratio of virtually any diagnostic assay. We therefore envision that this approach can be productively applied to a wide variety of research and clinical problems.

Genes of interest are typically those which have been found to be involved in inherited diseases. These include genes involved in colon cancer, breast cancer, Li-Fraumeni disease, cystic fibrosis, neurofibromatosis type 2, von Hippel-Lindau disease, as well as others. The identified genes include *APC*, *merlin*, *CF*, *VHL*, *hMSH2*, *p53*, *hPMS2*, *hMLH1*, *BRAC1*, as well as others. Mutations which can be identified at the protein level include those in sequences that regulate transcription or translation, nonsense mutations, splice site alterations, translocations, deletions, and insertions, or any other changes that result in substantial reduction of the full-length protein. Other subtler mutations can be detected at the nucleic acid level, such as by sequencing of RT-PCR products.

Cells of the human which may be used in fusions are any which can be readily fused to rodent cells. Peripheral blood lymphocytes (PBL) which are readily available clinical specimens are good fusion partners, with or without prior mitogenetic stimulation, whether used fresh or stored for over one year at -80° C. Since inherited mutations are the subject of the present method, any cells of the human body can be used, since all such cells contain essentially the same genetic complement.

Rodent cell recipients for fusion are preferably diploid, more preferably oncogene-transformed, and even more preferably have microsatellite instability due to a defect in a mismatch repair gene. Selection of particular clones which grow robustly, are stably diploid, and fuse at a high rate is well within the skill of the ordinary artisan.

Fusion of cells according to the present invention can be accomplished according to any means known in the art. Known techniques for inducing fusion include polyethylene glycol-mediated fusion, Sendai virus-mediated fusion, and electro-fusion. Cells can desirably be mixed at a ratio of between 10:1 and 1:10

human to rodent. Clones of fused cells generally become visible after about two to three weeks of growth.

Fused hybrid cells can be selected using any markers which result in a positively selectable phenotype. These include antibiotic resistance genes, toxic metabolite resistance genes, prototrophic markers, etc. The surprising advantage of the present invention is that a single marker on a single human chromosome can be used in the selection, and that stable hybrids containing more than just the single, selected human chromosome result. Thus markers on other chromosomes can be analyzed even when the chromosomes on which the markers reside were not selected.

Fused hybrid cells can be analyzed to determine that they do in fact carry a human chromosome which carries a gene of interest. Hybrid cells which have either of the two relevant human chromosomes can be distinguished from each other as well as from hybrids which contain both of the two human chromosomes. See **Fig. 1.** While any means known in the art for identifying the human chromosomes can be used, a facile analysis can be performed by assessing microsatellite markers on the human chromosome. Other linked polymorphic markers can be used to identify a desired human chromosome in the hybrids.

Once hybrid cells are isolated which contain one copy of a human gene of interest from a human who is being tested, mutation analysis can be performed on the hybrid cells. The genes can be tested directly for mutations, or alternatively the mRNA or protein products of the genes can be tested. Mutations that result in reduced expression of the full-length gene product should be detectable by Western blotting using appropriate antibodies. Tests which rely on the function of the protein encoded by the gene of interest and enzyme assays can also be performed to detect mutations. Other immunological techniques can also be employed, as are known in the art.

If an immunological method is used to detect the protein product of the gene of interest in the hybrids, it is desirable that antibodies be used that do not cross-react with rodent proteins. Alternatively, the rodent genes which are homologous to the gene of interest can be inactivated by mutation to simplify the analysis of protein products. Such mutations can be achieved by targeted mutagenesis methods, as is well known in the art.

Functional tests can also be used to assess the normalcy of each allelic product. For example, if one inserted an expression construct comprising a β-galactosidase gene downstream from a p53 transcriptional activation site, into a rodent-human hybrid cell that contained human chromosome 17 but no endogenous p53, then one could detect mutations of the p53 on the human chromosome 17 by staining clones with X-gal. Other enzymatic or functional assays can be designed specifically tailored to the gene of interest.

Any method of detecting mutations at the DNA or RNA level as are known in the art may be employed. These include without limitation, sequencing, allele-specific PCR, allele-specific hybridization, microarrays, DGGE, and automated sequencing.

It is a possibility that expression of the gene of interest might be inhibited in the hybrid cell environment. In order for the loss of expression of a gene of interest in the hybrid cells to be meaningfully interpreted as indicating a mutation in the human, one must confirm that the gene of interest, when wild-type, is expressed in rodent-human hybrid cells. This confirmation need not be done for each patient, but can be done once when the assay is being established.

When the assay of the present invention indicates that a mutation exists in the gene of interest, other family members can be tested to ascertain whether they too carry the mutation. Alternatively, the other family members can be tested to see if they carry the same chromosome as the affected family member. This can be determined by testing for a haplotype, i.e., a set of distinctive markers which are found on the chromosome carrying the mutation in the affected family member. Determination of a haplotype is a by-product of performing the assay of the invention on the first family member. When the hybrid cells are tested to confirm the presence of the relevant chromosome in the hybrid, for example by use of microsatellite markers, a distinctive marker set will be identified, which can then be used as a haplotype.

Mixed populations of hybrid cells made by the fusion process of the present invention may contain hybrid cells which are haploid for a number of different human chromosomes. Typically each homolog of at least 2, at least 5, at least 10, at least 15, at least 20, or even 22 human autosomes will be present in the population in a haploid

condition in at least one out of one hundred, seventy-five, fifty, thirty or twenty-eight of the cells. Thus a high proportion of the cells contain multiple human chromosomes, and a relatively small number of cells must be tested to find cells harboring a single copy of a non-selected chromosome.

Populations of cells resulting from a single hybrid are uniform and homogeneous due to the high stability of the human chromosomes in the hybrid cells of the invention. Thus at least 75%, 80%, 85%, 90%, 95%, 97%, 99%, or 100% of the cells in the population resulting from a single hybrid cell contain the same complement of human chromosomes.

The following examples provide experimental details which demonstrate one of many ways to carry out the invention. The invention is not limited to the particular methods of cells employed in the examples. The claims and the specification as a whole provide the measure of the invention.

Examples

Example 1

An outline of the approach is presented in Fig. 1. The rodent fusion partner was a line derived from mouse embryonic fibroblasts transformed with ras and adenovirus E1A oncogenes. HPRT-deficient subclones of this line were generated, and one subclone (E2) was chosen for further experimentation based on its robust growth characteristics, maintenance of diploidy, and fusion efficiency (10). Human lymphocytes cells were mixed with E2 cells at an optimum ratio and electrofused, and hybrids selected in geneticin (to kill unfused human cells) and HAT (to kill unfused E2 cells) (11). Colonies appearing after two weeks of growth were expanded and RNA and DNA prepared for analysis. From a single fusion experiment, an average of 36 hybrid clones were obtained (range of 17 to 80 in five different individuals).

All hybrids contained the human X chromosome, as this chromosome contains the HPRT gene allowing growth in HAT. To determine whether other human chromosomes were present in the hybrids, polymorphic microsatellite markers (12) were used as probes in PCR-based assays (Fig. 2A). We focused on the chromosome arms (2p, 2q, 3p, 5q, 7q, and 16q) known to contain colorectal cancer (CRC) predisposition genes. One copy of each of these chromosome arms was present in a significant fraction of the hybrid clones. For example, of 476 hybrids derived from

14 individuals and examined for chromosome 3, 136 hybrids contained neither donor chromosome, 211 hybrids contained both donor chromosomes, 60 hybrids contained one parent's chromosome, and 69 hybrids contained the other parent's chromosome. Similar retention frequencies were found for all six chromosome arms analyzed. Testing of markers from both arms of a single chromosome showed that whole chromosomes, rather than chromosome fragments, were generally retained in the hybrids. This result was confirmed with fluorescence in situ hybridization (FISH) on metaphase spreads from the hybrids, which indicated the presence of 11 ±3 human chromosomes in each hybrid cell. Calculations based on the genotypic data indicated that the analysis of 25 hybrids would ensure a 95% probability of identifying at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of a single chromosome under study. Moreover, it would require only 45 hybrids to similarly ensure that each allele of all 22 autosomes was present and separated from its homolog in at least one hybrid (13).

Example 2

Two other features of the hybrids were essential for the analyses described below. First, the human chromosome complements of the hybrids were remarkably stable. Polymorphic marker analysis in ten hybrids revealed identical patterns of retention after growth for 90 (30 passages) generations after initial genotyping. Second, those hybrids containing the relevant chromosome expressed every human gene assessed, including all known colorectal cancer susceptibility genes (the *hMSH2* and *hMSH6* genes on chromosome 2p, the *hPMS1* gene on chromosome 2q, the *TGF-β Receptor Type II* gene and *hMLH1* gene on chromosome 3p, the *APC* gene on chromosome 5q, the *hPMS2* gene on chromosome 7q, and the *E-cadherin* gene on chromosome 16q; representative examples in Fig. 2B) (14).

Example 3

Having established the stability and expression patterns of CRC-predisposition genes in these hybrids, we used this "conversion" approach to investigate ten patients who had proven refractory to standard genetic diagnostic techniques. Each of these patients had a significant family history of colorectal cancer and evidence of mismatch repair deficiency in their tumors, yet sequencing of the entire coding sequence of each

known MMR gene had failed to reveal mutations. Indeed, these and similar studies have prompted the speculation that other major HNPCC genes must exist. (25-34) Hybrids were generated from lymphocytes of each patient, and at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of each MMR gene was isolated. Analysis of the nucleic acids from these hybrids revealed specific mutations in all ten patients (Table 1). In every case, an abnormality was found in a single allele of either *hMSH2* or *hMLH1*. The nature of the abnormalities revealed why they had not been detected with the standard methods previously used for their analysis. Three cases were due to large deletions, encompassing six or seven exons. When corresponding nucleic acids from the cells of such patients are evaluated by any PCR-based method, only the wild type sequences from the unaffected parent would be amplified, leading to the false impression of normalcy (for example, case #1 in Figure 3). Though Southern blotting can reveal deletions of one or a few exons in MMR, larger deletions are refractory to such blotting methods. In three cases (#4, 6, and 9), no transcript was generated from one allele, though the sequences of all exons and intron-exon borders from this allele were normal. Presumably, mutations deep within an intron or within the promoter of the gene were responsible. The absence of transcripts from one specific allele of these three patients was confirmed in at least three other converted hybrids from each patient. In four other cases, point mutations were found (Table 1). These mutations were not detected in the original sequence analyses because the signals from the mutant allele were not as robust as those from the wild type. Such asymmetry can be caused by instability of mutant transcripts due to nonsense mediated decay (36-38), or to nucleotide preferences of the polymerases in specific sequence contexts, and represents a common problem for both manual and automated sequencing methods (39). The conversion approach eliminates these problems because only one sequence can possibly be present at each position. A good example of this was provided by Warthin G (17). The mutation in this prototype kindred was an A to C transversion at a splice site. The signal from the mutant "C" in the sequencing ladder was not as intense as the wild type "A" (Fig. 4b). This mutation led to the use of a cryptic splice site 24 bp upstream of exon 4, and an under-represented transcript with a 24 base insertion (Fig. 4a). To demonstrate that this mutation had an effect at the protein level, we analyzed the hybrids by

immunoblotting with specific antibodies (19). The hybrids containing the mutant allele did not make detectable levels of human hMSH2 protein, though they did synthesize normal levels of a control protein (Fig. 4C).

The results described above demonstrate that individual alleles of human chromosomes can be readily isolated upon fusion to mouse cells.

HNPCC provides a cogent demonstration of the power of the conversion approach because it is a common genetic disease that has been widely studied. In the last three years, for example, extensive analyses of the major MMR genes have been performed in 303 HNPCC kindreds from nine cohorts distributed throughout the world (25-34). Based on the fraction of such patients with characteristic microsatellite instability in their cancers (30-34), it can be estimated that 239 (78%) of the kindreds had germ-line mutations of mismatch repair genes. Yet MMR gene mutations were identified in only 127 (42%) of these 239 kindreds (25-34). Our cohort was similar, in that it was derived from a total of 25 kindreds, 22 of whom had tumors with microsatellite instability and presumptive MMR gene mutations. Of these 22, our initial analyses revealed mutations in only 12 (54%) (ref. 14 and unpublished data). Mutations of the other ten patients were only revealed upon conversion analysis, which thereby increased the sensitivity from 54% to 100%. The conclusion that virtually all cases of HNPCC associated with MSI are due to germline mutations of known MMR genes is consistent with recent immunohistochemical data demonstrating the absence of either MSH2 or MLH1 protein staining in the cancers from the great majority of HNPCC patients (40, 41). A corollary of these results is that the search for new human MMR genes should not based on the premise that a large fraction of HNPCC cases will prove attributable to such unknown genes.

The system described above can be applied to other genetic diseases in a straight forward manner. It should be emphasized that this approach is not a substitute for the many powerful methods currently available to search for specific mutations. Rather, conversion can be used to maximize the sensitivity of existing techniques. Converted nucleic acids provide the preferred substrates for such methods because of the higher signal to noise attainable and the inability of the wild type allele to mask or confound detection of the mutant allele. As DNA-based mutational assays are

improved in the future, and progressive incorporate microarrays and other automatable features (42-44), the value of conversion-generated nucleic acids will correspondingly increase, significantly enhancing the effectiveness of genetic tests for hereditary disease.

Methods

Cell culture

Mouse embryonic fibroblasts were derived from MSH2-deficient mice (46) and transformed with adenovirus E1A and RAS oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

Cell fusion and the generation of hybrids

The patients were from kindreds with HNPCC as defined by the Amsterdam criteria (44); in no case was linkage analysis feasible due to the lack of a sufficient number of affected individuals. Microsatellite instability (MSI) in the cancers from these patients was determined through the markers recommended in ref. 45. 3 x 10^6 E2 cells and 12 x 10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX ElectroCell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersburg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. From a single fusion, an average of 23 +/- 15 hybrid clones were obtained. The lymphocytes used for the experiments described here were derived from Epstein-Barr Virus infection of peripheral blood leukocytes, but it was found that freshly drawn lymphocytes could also be successfully fused and analyzed using identical methods.

Genotyping

Genotyping was performed as described (12). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788 and D2S1360, D2S1384, D3S2406, D7S1824, and D16S3095, from chromosome 2p, 2q, 3p, 5q, 7q and 16q, respectively. Fluorescence in situ hybridization was performed as described previously (21).

PCR and sequencing

Polyadenylated RNA was purified and RT-PCR performed as described previously. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through an internet site.

Statistical analysis

the number of hybrids containing none, both or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the number of hybrids required to generate mono-allelic hybrids containing specific numbers of each chromosomes.

Table 1 Summary of MMR Gene Mutations Identified by Conversion

Kindred	Gene	Functional abnormality	Genomic DNA alteration
1	MSH2	No detectable transcript	Deletion of exon 1-exon 6
2	MSH2	R680Ter	CGA to TGA
3	MSH2	No detectable transcript	Deletion of exon 1-exon 7
4	MLH1	No detectable transcript	NI*
5	MSH2	A636P**	GCA to CCA
6	MLH1	No detectable transcript	NI*
7	MSH2	R680Ter	CGA to TGA
8	MSH2	No detectable transcript	Deletion of exon 1-exon 7
9	MLH1	No detectable transcript	NI*
10	MSH2	24 bp insertion between codons 215 and 216	TAG to GAG, splice acceptor of exon 4

*Genomic alteration not identified despite complete sequencing of all exons and intron-exon borders of the abnormal allele.

**Found in other HNPCC kindreds, but not in 400 normal alleles (ref. 46 and unpublished data); mutant product functionally defective (ref. 46).

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10. E2 cells were derived from mouse embryonic fibroblasts derived from MSH2-deficient mice (generously provided by T. Mak) and transformed with adenovirus *E1A* and *RAS* oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were

[1]All references are explicitly incorporated by reference.

maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

11. 3×10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX Electro Cell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersberg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. The lymphocytes used for the experiments described here were derived from Epstein-Barr virus infection of peripheral blood leukocytes, but we found that freshly drawn lymphocytes could also be successfuly fused and analyzed using identical methods

12. Genotyping was performed as described in F.S. Leach et al., *Cell* 75, 1215 (1993). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788, D2S13360, D3S2406, D7S1824, and D16S3095, from chromosomes 2p, 2q, 3p, 5q, and 16q, respectively.

13. The numbers of hybrids containing none, both, or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the numbers of hybrids required to generate mono-allelic hybrids containing specific numbers of chromosomes.

14. Polyadenylated RNA was purified and RT-PCR performed as described in B. Liu et al., *Nat Medicine* **2**, 169 (1996).

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18. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through a *Science* internet site.

19. Cytoplasmic extracts of cells hybrids were separated by electrophoresis through SDS-polyacrylamide gels and immunoblotted with antibodies specific for human hMSH2 (#NA26, Calboiochem), human hMLH1 (#13271A, Pharmingen), or β-Tubulin (#N357, Amersham).

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CLAIMS

1. A method of detecting mutations in a gene of interest of a human, comprising the steps of:

fusing cells of a human to rodent cell recipients to form human-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human chromosome, to form a population of fused cell hybrids;

detecting among the population of fused cell hybrids a subset of hybrids which are haploid for a second human chromosome which is not the same chromosome as the first human chromosome and which was not selected;

testing said subset of hybrids to detect an mRNA product of a gene or a protein product of said gene, wherein the gene resides on the second human chromosome, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product indicate the presence of a mutation in the gene in the human.

2. The method of claim 1 wherein the rodent cell is diploid.

3. The method of claim 1 wherein the rodent cell recipients are deficient in mismatch repair.

4. The method of claim 1 wherein the rodent cell recipient is geneticin resistant.

5. The method of claim 1 wherein the rodent cell recipients are transformed with an oncogene.

6. The method of claim 5 wherein the oncogene is *ras*.

7. The method of claim 5 wherein the oncogene is *E1A*.

8. The method of claim 1 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

9. The method of claim 1 wherein the rodent cell recipients are *MSH2*$^-$.

10. The method of claim 1 wherein the rodent cell recipient is diploid, and contains both a selectable marker and a counterselectable marker.

11. The method of claim 10 wherein the counterselectable marker is HPRT deficiency.

12. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third human chromosome, wherein the first, second, and third human chromosomes are distinct, and neither the second nor the third human chromsomes were selected.

13. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third and fourth human chromosome, wherein the first through fourth human chromosomes are distinct, and neither the second through fourth human chromsomes were selected.

14. The method of claim 12 further comprising the step of testing said subset of hybrids to detect an mRNA product of a second gene or a protein product of said second gene, wherein the second gene resides on the third human chromosome, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product from said second gene indicate the presence of a mutation in the second gene in the human.

15. The method of claim 13 testing said subset of hybrids to detect an mRNA product of a second and third gene or a protein product of said second and third gene, wherein the second and third genes reside on the third and fourth human chromosomes, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product of said third or fourth genes indicate the presence of a mutation in third or fourth genes in the human.

16. The method of claim 1 wherein the cells of the human are lymphocytes.

17. The method of claim 1 wherein the step of detecting a subset of hybrids which are haploid for the second human chromosome is accomplished by identifying the presence of a microsatellite marker on the second human chromosome.

18. A method of providing test cells for genetic testing, wherein said test cells are haploid for human genes, comprising the steps of:

fusing cells of a human to transformed, diploid, rodent cell recipients to form human-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a marker on each of a first human chromosome and a rodent chromosome, whereby a population

of cells are formed which stably maintain one or more human chromosomes in the absence of selection for the human chromosomes;

detecting among the population of cells those cells which are haploid for a second and third human chromosome which are distinct from the first human chromosome and which were not selected.

19. The method of claim 18 wherein the rodent cell recipient is geneticin resistant.

20. The method of claim 18 wherein the rodent cell recipients are transformed with an oncogene.

21. The method of claim 20 wherein the oncogene is *ras*.

22. The method of claim 20 wherein the oncogene is *E1A*.

23. The method of claim 18 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

24. The method of claim 18 wherein the rodent cell recipients are deficient in mismatch repair.

25. The method of claim 18 wherein the rodent cell recipient contains a counterselectable marker.

26. The method of claim 25 wherein the counterselectable marker is HPRT deficiency.

27. The method of claim 18 wherein the cells of the human are lymphocytes.

28. The method of claim 18 wherein the step of detecting cells which are haploid for said second and third human chromosomes is performed by identifying the presence of a microsatellite marker on said second and third human chromosomes.

29. The method of claim 18 further comprising the step of:

detecting among the population of cells, cells haploid for a fourth human chromosome which is distinct from the first, second, and third human chromosomes and which was not selected.

30. The method of claim 18 futher comprising the step of:

detecting among the population of cells, cells haploid for a fourth, fifth, and sixth human chromosome, wherein said fourth through sixth human

chromosomes are distinct from the first, second and third human chromosomes and were not selected.

31. The method of claim 18 further comprising:

testing nucleic acids of a cell haploid for the third human chromosome for a mutation in a gene on the third human chromosome.

32. The method of claim 18 further comprising:

testing proteins of a cell haploid for the second and third human chromosome for a mutation in a gene on each of the second and third human chromosomes.

33. The method of claim 1 wherein mRNA of a cell in the subset of hybrids is tested for a mutation in a gene on the second human chromosome.

34. The method of claim 1 wherein proteins of a cell in the subset of hybrids are tested for a mutation in a gene on the second human chromosome.

35. A population of rodent-human hybrid cells wherein each homolog of at least 2 human autosomes is present in haploid form in at least one out of one hundred of the cells.

36. The population of claim 35 wherein each homolog of at least 5 human autosomes is present in haploid form in at least one out of a hundred of the cells.

37. The population of claim 35 wherein each homolog of at least 5 human autosomes is present in haploid form in at least one out of fifty of the cells.

38. The population of claim 35 wherein each homolog of at least 5 human autosomes is present in haploid form in at least one out of thirty of the cells.

39. The population of claim 35 wherein each homolog of at least 10 human autosomes is present in haploid form in at least one out of a hundred of the cells.

40. The population of claim 35 wherein each homolog of at least 10 human autosomes is present in haploid form in at least one out of fifty of the cells.

41. The population of claim 35 wherein each homolog of at least 10 human autosomes is present in haploid form in at least one out of a thirty of the cells.

42. The population of claim 35 wherein each homolog of at least 15 human autosomes is present in haploid form in at least one out of a hundred of the cells.

43. The population of claim 35 wherein each homolog of at least 15 human autosomes is present in haploid form in at least one out of fifty of the cells.

44. The population of claim 35 wherein each homolog of at least 15 human autosomes is present in haploid form in at least one out of thirty of the cells.

45. The population of claim 35 wherein each homolog of at least 20 human autosomes is present in haploid form in at least one out of a hundred of the cells.

46. The population of claim 35 wherein each homolog of at least 20 human autosomes is present in haploid form in at least one out of fifty of the cells.

47. The population of claim 35 wherein each homolog of at least 20 human autosomes is present in haploid form in at least one out of thirty of the cells.

48. The population of claim 35 wherein each homolog of at least 22 human autosomes is present in haploid form in at least one out of a hundred of the cells.

49. The population of claim 35 wherein each homolog of at least 22 human autosomes is present in haploid form in at least one out of fifty of the cells.

50. The population of claim 35 wherein each homolog of at least 22 human autosomes is present in haploid form in at least one out of thirty of the cells.

51. A population of rodent-human hybrid cells which stably maintain their human chromosome content such that at least 95% of the hybrid cells contain the same human chromosomes.

52. The population of rodent-human hybrid cells of claim 51 whererein at least 97% of the hybrid cells contain the same human chromosomes.

53. The population of rodent-human hybrid cells of claim 51 whererein at least 99% of the hybrid cells contain the same human chromosomes.

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

ABSTRACT OF THE DISCLOSURE

Detection of mutations associated with hereditary diseases is complicated by the diploid nature of human cells. Mutations present in one allele are often masked by the wild-type sequence of the other allele. Individual alleles can be isolated from every chromosome within somatic cell hybrids generated from a single fusion. Nucleic acids from the hybrids can be analyzed for mutations in an unambiguous manner. This approach was used to detect two cancer-causing mutations that had previously defied genetic diagnosis. One of the families studied, Warthin Family G, was the first kindred with a hereditary colon cancer syndrome described in the biomedical literature.

SAK

FILING RECEIPT

OC000000005254877

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: ASSISTANT SECRETARY AND
COMMISSIONER OF PATENT AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
09/504,860	02/16/2000	1643	812	04993.86286	4	36	5

Banner & Witcoff Ltd
1001 G Street NW
Washington, DC 20001-4597

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JUL 25 2000

BANNER & WITCOFF LTD.

Date Mailed: 07/20/2000

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Customer Service Center. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the PTO processes the reply to the Notice, the PTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Bert Vogelstein, Baltimore, MD ;
Kenneth W. Kinzler, BelAir, MD ;
Nickolas Papadopoulos, New York, NY ;

Continuing Data as Claimed by Applicant

THIS APPLICATION IS A CIP OF 09/461,047 12/15/1999
WHICH CLAIMS BENEFIT OF 60/158,160 10/08/1999

0/10 7,862 86
DOCKETED ans
Filing Receipt
JUL 25 2000
Recvd.

Foreign Applications

If Required, Foreign Filing License Granted 04/18/2000

**** SMALL ENTITY ****

Title

Converting diploidy to haploidy for genetic diagnosis

Preliminary Class

435

Data entry by : JACKSON, MINNIE **Team :** OIPE **Date:** 07/20/2000

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CRF 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 36 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Office of Export Administration, Department of Commerce (15 CFR 370.10 (j)); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

PLEASE NOTE the following information about the Filing Receipt:

- The articles such as "**a**," "**an**" and "**the**" are not included as the first words in the title of an application. They are considered to be unnecessary to the understanding of the title.
- The words "***new***," "***improved***," "***improvements in***" *or* "***relating to***" are not included as first words in the title of an application because a patent application, by nature, is a new idea or improvement.
- The title may be truncated if it consists of more than 600 characters (letters and spaces combined).
- The docket number allows a maximum of 25 characters.
- If your application was submitted under 37 CFR 1.10, your filing date should be the "date in" found on the Express Mail label. If there is a discrepancy, you should submit a request for a corrected Filing Receipt along with a copy of the Express Mail label showing the "date in."

Any corrections that may need to be done to your Filing Receipt should be directed to:

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Washington, DC 20231

Applicant or Patentee: Bert Voelstein, Kenneth W. Kinzler, and Nickolas Papadopoulos Attorney's #: 01107.86286
Serial or Patent No.: 09/504,860 Filed or Issued: February 16, 2000
For: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§1.9(f) AND 1.27(c)) — SMALL BUSINESS CONCERN

I hereby declare that I am:

- ☐ the owner of the small business concern identified below;
- ■ an official of the small business concern empowered to act on behalf of the concern identified below;

NAME OF CONCERN: GMP Genetics, Inc.
ADDRESS OF CONCERN: One East Broward Boulevard, Suite 1701
Fort Lauderdale, FL 33301

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR §§121.3-18, and reproduced in 37 CFR §1.9(d), for purposes of paying reduced fees under §§41(a) and (b) of Title 35, United States Code, in that the number of employees of the business concern (including those of its affiliates) does not exceed 500 persons. For purposes of this Statement: (1) the number of employees of the business concern is the average over the previous fiscal year of the business concern of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year; and (2) business concerns are affiliates of each other when either directly or indirectly one business concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS
by inventor(s) Bert Vogelstein, Kenneth W. Kinzler and Nickolas Papadopoulos
described in
- ☐ the specification filed herewith.
- ■ application serial no. 09/504,860 filed February 16, 2000
- ☐ patent no. _____ filed _____

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR §1.9(c) or by any concern which would not qualify as a small business concern under 37 C.F.R. §1.9(d) or a nonprofit organization under 37 C.F.R. §1.9(e). *NOTE: Separate verified statements are required from each named person, business concern or organization having rights to the invention averring to their status as small entities. (37 CFR §1.27)

FULL NAME Johns Hopkins University	☐	Individual
ADDRESS 111 Market Place, Suite 906	☐	Small Business Concern
Baltimore, MD 21201	■	Nonprofit Organization
FULL NAME _____	☐	Individual
ADDRESS _____	☐	Small Business Concern
_____	☐	Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR §1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States Code; and further that false statements made willfully may jeopardize the validity of the application, of any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Bart Chernow, M.D., President
TITLE OF PERSON (other than owner) President
ADDRESS OF PERSON SIGNING ~~One East Broward Boulevard, Suite 1701~~
Fort Lauderdale, FL 33301

SIGNATURE _____ DATE 05/26/00

LAW OFFICES
BANNER & WITCOFF, LTD.
1001 G STREET, N.W.
WASHINGTON, D.C. 20001-4597
(202)508-9100

Applicant or Patentee: Bert Vogelstein, Kenneth W. Kinzler, and Nickolas Papadopoulos
Serial or Patent No.: 09/504,860
For: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

Attorney's #: 01107.86286
Filed or Issued: 02/16/2000

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§1.9(f) AND 1.27(d)) — NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:
NAME OF ORGANIZATION: The Johns Hopkins University
ADDRESS OF ORGANIZATION: 111 Market Place, Suite 906, Baltimore, Maryland 21201

TYPE OF ORGANIZATION

- ■ UNIVERSITY OF OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. §§501(a) and 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA (NAME OF STATE) _____)
  (CITATION OF STATUTE _____)
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 U.S.C. §§501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
  (NAME OF STATE _____)
  (CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e) for purposes of paying reduced fees under §§41(a) and (b) of Title 35, United States Code, with regard to the invention entitled CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS
by inventor(s) Bert Vogelstein, Kenneth W. Kinzler, and Nickolas Papadopoulos
described in
- ☐ the specification filed herewith.
- ■ application serial no. 09/504,860 filed 02/16/2000
- ☐ patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d) or by any concern which would not qualify as a small business concern under 37 C.F.R. §1.9(d) or a nonprofit organization under 37 C.F.R. §1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. §1.27)

FULL NAME GMP Genetics, Inc	☐	Individual
ADDRESS One East Broward Boulevard, Suite 1701	■	Small Business Concern
Fort Lauderdale, FL 33301	☐	Nonprofit Organization
FULL NAME _____	☐	Individual
ADDRESS _____	☐	Small Business Concern
_____	☐	Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR §1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States Code; and further that false statements made willfully may jeopardize the validity of the application, of any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Nina Ossanna, Ph.D.
TITLE IN ORGANIZATION Director, Office of Technology Licensing
ADDRESS OF PERSON SIGNING 111 Market Place #906, Baltimore, MD 21202

SIGNATURE _____ DATE 6/8/00

LAW OFFICES
BANNER & WITCOFF, LTD.
1001 G STREET, N.W.
WASHINGTON, D.C. 20001-4597
(202)508-9100

JOINT DECLARATION FOR PATENT APPLICATION

As the below named inventor, we hereby declare that:

Our residence, post office address and citizenship are as stated below next to our names;

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS, the specification of which

- ☐ is attached hereto.
- ■ was filed on February 16, 2000 as Application Serial Number 09/504,860 and was amended on (if applicable).
- ☐ was filed under the Patent Cooperation Treaty (PCT) and accorded International Application No. _____, filed _____, and amended on _____ (if any).

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We hereby acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Prior Foreign Application(s)

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application No.	Date of Filing (day month year)	Date of Issue (day month year)	Priority Claimed Under 35 U.S.C. §119

Prior United States Provisional Application(s)

We hereby claim priority benefits under Title 35, United States Code, §119(e)(1) of any U.S. provisional application listed below:

U.S. Provisional Application No.	Date of Filing (day month year)	Priority Claimed Under 35 U.S.C. §119(e)(1)
60/158,160	08 October 2000	Yes

Prior United States Application(s)

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Date of Filing (Day, Month, Year)	Status — Patented, Pending, Abandoned
09/461,047	15 December 1999	Yes

Power of Attorney

And we hereby appoint, both jointly and severally, as our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys and agents, their registration numbers being listed after their names:

ALTHERR, Robert F.	31,810	HONG, Patricia E.	34,373	PATEL, Binal J.	42,065
BANNER, Donald W.	17,037	HOSCHEIT, Dale H.	19,090	PATHAK, Ajay S.	38,266
BANNER, Mark T.	29,888	IWANICKI, John P.	34,628	PAYNE, Stephen S.	35,316
BANNER, Pamela I.	33,644	JACKSON, Thomas H.	29,808	PETERSON, Thomas L.	30,969
BECKETT, William W.	18,262	KAGAN, Sarah A.	32,141	POTENZA, Joseph M.	28,175
BODNER, Jordan	42,338	KATZ, Robert S.	36,402	PRATT, Thomas K.	37,210
BUROW, Scott A.	42,373	KLEIN, William J.	43,719	RENK, Christopher J.	33,761
CALLAHAN, James V.	20,095	KRAUSE, Joseph P.	32,578	RESIS, Robert H.	32,168
CHANG, Steve S	42,402	LINEK, Ernest V.	29,822	RIVARD, Paul M.	43,446
COHAN, Gregory J.	40,959	MAGOON, Sumeet	43,769	SCHAD, Steve P.	32,550
COOPERMAN, Marc S.	34,143	MALONE, Dale A.	32,155	SHANAHAN, Michael H.	24,438
CURTIN, Joseph P.	34,571	MANNAVA, Ashok K.	45,301	SHIFLEY, Charles W.	28,042
DAWSON, John R.	39,504	McDERMOTT, Peter D.	29,411	SKERPON, Joseph M.	29,864
DEMOOR, Laura J.	39,654	McKEE, Christopher L.	32,384	SPAR, Elizabeth	45,123
EVANS, Thomas L.	35,805	McKIE, Edward F.	17,335	STOCKLEY, D. J.	34,257
FEDOROCHKO, Gary D.	35,509	MEDLOCK, Nina L.	29,673	VAN ES, J. Pieter	37,746
FISHER, Daniel E.	34,162	MEECE, Timothy C.	38,553	WILLIAMS, Kathleen M.	34,380
FISHER, William J.	32,133	MEEKER, Frederic M.	35,282	WITCOFF, Sheldon W.	17,399
GLEMBOCKI, Christopher R.	38,800	MILLER, Charles L.	43,805	WOLFF, Kevin A.	42,233
HANLON, Brian E.	40,449	MITRIUS, Janice V.	43,808	WOLFFE, Franklin D.	19,724
HEMMENDINGER, Lisa M.	42,653	MORENO, Christopher P.	38,566	WOLFFE, Susan A.	33,568
HILLMAN, Lisa	43,673	NELSON, Jon O.	24,566	WRIGHT, Bradley C.	38,061
		NIEGOWSKI, James A.	28,331		

All correspondence and telephone communications should be addressed to:

Banner & Witcoff, Ltd.
1001 G Street, N.W., 11th Floor
Washington, D.C. 20001-4597

Customer Number: 22907
Tel: (202) 508-9100
Fax: (202) 508-9299

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature _____ Date 6/12/02

Full Name of First Inventor Vogelstein (Family Name) Bert (First Given Name) (Second Given Name)

Residence Baltimore, Maryland Citizenship United States

Post Office Address 3700 Breton Way, Baltimore, Maryland 21208

Signature _____ Date 6/13/02

Full Name of Second Inventor Kinzler (Family Name) Kenneth (First Given Name) W. (Second Given Name)

Residence BelAir, Maryland Citizenship United States

Post Office Address 1403 Halkirk Way, BelAir, Maryland 21015

Signature N Papadopoulos Date 6/22/00

Full Name of Third Inventor Papadopoulos Nickolas
Family Name First Given Name Second Given Name

Residence New York, New York Citizenship United States

Post Office Address 90 Morningside Drive #2F, New York, New York 10027

RECORDATION FORM COVER SHEET

PATENTS ONLY

Form PTO-1592 (modified)

01107.86286

To the Honorable Commissioner of Patents and Trademarks: Please record the attached original documents or copies thereof:

1. Name of Conveying Party or Parties:

Bert Vogelstein
Kenneth W. Kinzler
Nicholas Papadopoulos

Additional Names of conveying party or parties attached?
☐ Yes ■ No

2. Name and address of receiving party or parties:

The Johns Hopkins University
720 Rutland Avenue
Baltimore, Maryland 21205

Additional Name(s) and address(es) attached? ☐ Yes ■ No

3. Nature of Conveyance:

■ Assignment ☐ Merger

☐ Security Agreement ☐ Change of Name

☐ Other _____

Execution Date: June 13 and 22, 2000

4. Application Number(s) or Patent Number(s):

If this document is being filed together with a new application, the execution date of the application is: _____

A. Patent Application Number(s):

09/504,860

Additional Name(s) and address(es) attached? ☐ Yes ■ No

B. Patent Number(s):

5. Name and address of party to whom correspondence concerning the document should be mailed:

Banner & Witcoff, Ltd.
1001 G Street, N.W., Suite 1100
Washington, D.C. 20011

6. Total Number of Applications and patents involved: 1

7. Total fee (37 CFR 3.41) ..... $ 40.00

■ Enclosed
☐ Authorized to be charged to deposit account
■ Please charge or credit our deposit account for any additional or refunded fees associated with recording this assignment

8. Deposit Account No.:
19-0733 (in the event additional fees are required)

(Duplicate copy of this page attached if paying by deposit account)

Do Not Use this Space

9. Statement and Signature:

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Sarah A. Kagan, Reg. No. 32,141 _____ July 12, 2000
Name of Person Signing Signature Date

Total Number of Pages comprising Cover Sheet: 3

ASSIGNMENT

WHEREAS, WE, Bert Vogelstein, citizen of the United States, residing at 3700 Breton Way, Baltimore, Maryland 21208; Kenneth W. Kinzler, citizen of the United States, residing at 1403 Halkirk Way, BelAir, Maryland 21015; and Nickolas Papadopoulos, citizen of the United States, residing at 90 Morningside Drive #2F, New York, New York 10027, have invented certain new and useful improvements in **"CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS"**, for which an application for Letters Patent of the United States was filed on February 16, 2000, and accorded Serial No. 09/504,860; and

WHEREAS, The Johns Hopkins University, a corporation duly organized under the laws of the State of Maryland having a place of business in Baltimore, Maryland, is desirous of acquiring the entire right, title and interest in and to the aforesaid invention and in and to any Letters Patent of the United States or any foreign country which may be granted therefor;

NOW THEREFORE, for good and valuable consideration, we, the said Bert Vogelstein, Kenneth W. Kinzler, and Nickolas Papadopoulos, by these presents do sell, assign, and transfer unto The Johns Hopkins University, its successors, legal representatives and assigns, the full and exclusive right to the said invention as described in the said application, and the entire right, title and interest in and to any and all Letters Patent which may be granted therefor in the United States and its territorial possessions and in any and all foreign countries and in and to any and all divisions, reissues, continuations and extensions thereof;

AND WE HEREBY authorize and request the Commissioner of Patents and Trademarks or any other proper officer or agency of any country to issue all said Letters Patent to said assignee;

AND WE HEREBY warrant and covenant that we have full right to convey the entire interest herein assigned and that we have not executed and will not execute any instrument or assignment in conflict herewith;

AND WE HEREBY agree to communicate to said assignee or its representatives any facts known to us respecting said invention, to execute all divisional, continuation, reissue and foreign applications, sign all lawful documents and make all rightful oaths relating to said invention, and to testify in any judicial or administrative proceeding and generally do everything possible to aid the said assignee to obtain and enforce said Letters Patent in the United States or any foreign country when requested so to do by said assignee.

IN WITNESS WHEREOF, we have hereunto set our hand and seal.

Date: 6/13/00 Inventor: _____

Bert Vogelstein

Date: 6/13/00 Inventor: _____

Kenneth W. Kinzler

Date: 6/22/00 Inventor: N Papadopoulos
Nickolas Papadopoulos

STATE OF MARYLAND)
City) ss
~~COUNTY~~ OF BALTIMORE)

On this 13th day of June, 2000 before me a Notary Public in and for the County and State aforesaid, personally appeared Bert Vogelstein, and Kenneth W. Kinzler, to me known and known to me to be the persons of those names, who signed and sealed the foregoing instrument, and acknowledged the same to be their free act and deed.

(S E A L) Barbara A. Lee
Notary Public

My Commission Expires: September 1, 2001

STATE OF NEW YORK)
) ss
COUNTY OF)

On this 22 day of June, 2000 before me a Notary Public in and for the County and State aforesaid, personally appeared Nickolas Papadopoulos, to me known and known to me to be the person of this name, who signed and sealed the foregoing instrument, and acknowledged the same to be their free act and deed.

(S E A L) Stella M. Franco
Notary Public

STELLA M. FRANCO
Notary Public, State of New York
No. 31-4804181
Qualified in New York County
Commission Expires 9/30/2000

My Commission Expires: September 30, 2000.

FORMALITIES LETTER

OC000000005059842

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENT AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/504,860	02/16/2000	Bert Vogelstein	04993.86286

Banner & Witcoff Ltd
1001 G Street NW
Washington, DC 20001-4597

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BANNER & WITCOFF LTD.

Date Mailed: 04/25/2000

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given TWO MONTHS from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
  Applicant must submit **$ 690** *to complete the basic filing fee and/or file a small entity statement claiming such status (37 CFR 1.27).*
- Total additional claim fee(s) for this application is $444.
  - **$288** for **16** total claims over 20.
  - **$156** for **2** independent claims over 3 .
- The oath or declaration is missing.
  A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(e) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.
- **The balance due by applicant is $ 1264.**

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APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
09/504,860	02/16/2000	1643	0	04993.86286	4	36	5

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Applicant(s)

Bert Vogelstein, Residence, NOT PROVIDED;

Continuing Data as Claimed by Applicant

THIS APPLICATION IS A CIP OF 09/461,047 12/15/1999
WHICH CLAIMS BENEFIT OF 60/158,160 10/08/1999

Foreign Applications

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Title

Converting diploidy to haploidy for genetic diagnosis

Preliminary Class

435

Data entry by : JACKSON, MINNIE **Team :** OIPE **Date:** 04/25/2000

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APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
09/504,860	02/16/2000	1643	0	04993.86286	4	36	5

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Applicant(s)

Bert Vogelstein, Residence, NOT PROVIDED;

amm alpha
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Continuing Data as Claimed by Applicant

THIS APPLICATION IS A CIP OF 09/461,047 12/15/1999
WHICH CLAIMS BENEFIT OF 60/158,160 10/08/1999

Foreign Applications

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Title

Converting diploidy to haploidy for genetic diagnosis

Preliminary Class

435

Data entry by : JACKSON, MINNIE **Team :** OIPE **Date:** 04/18/2000

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Serial No. TBA B&W # 04993.86286 Atty/Sec SAK/ama Date 2/16/2000

Inventor Bert Vogelstein Client GMP

Title Converting Diploidy to Haploidy for Genetic Diagnosis

The following has been received in the U.S. Patent and Trademark Office on the date stamped hereon:

☒ 27 total pp Spec., including : # of Claims 56 (# of independent claims 5); ☒ Abstract

☐ Foreign Priority on _____ Country, Appl. # and Date

☐ Priority on U.S. Prov. _____ B&W# _____

☐ Application : ☒ CIP ☐ Continuation ☐ Divisional Parent Ser. No. 09/461042 B&W# 01102.84952

☐ Provisional App ___ pp Spec/Clms; Cover Sheet

☐ Declaration/PoA : ☐ Executed ☐ Unexecuted

☒ Drawings : ☒ Formal ☐ Informal # of distinct sheets 4 : Figs. 14

☐ Assignment w/PTO Cover Sheet

☐ Small Entity Declaration

☐ IDS w/PTO 1449 ☐ Prior Art ☐ w/fee

☐ Preliminary Amendment

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☐ Request for Approval of Drawing Changes

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Applicant(s): Bert Vogelstein

jc598 U.S. PTO 09/504860 02/16/00

Title: Converting Diploidy to Haploidy for Genetic Diagnosis

Filing Date: 2/16/2000

Client: GMP

BBM&B No.: 04993.86286 Client Reference:

Attorney/Secretary: SAK/ama

Serial No.: TBA

3/91

UNITED STATES C(TINUING UTILITY PATENT PPLICATION
under 37 C.F.R. § 1.53(b)

Atty. Docket No. 04993.86286

Assistant Commissioner of Patents
Box Patent Applications
Washington, D.C. 20231

Enclosed herewith is a continuing patent application and the following papers:

First Named Inventor (or application identifier): Bert Vogelstein

Title of Invention: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

- ☐ Continuation
- ☐ Divisional
- ■ Continuation-in-Part

of prior application No. 09/461,047, filed December 15, 1999

1. ■ Specification 27 pages (including specification, claims, abstract) / 56 claims (5 independent)

2. ■ Declaration/Power of Attorney:
   - ☐ Copy from Prior Application (for continuation or divisional application)
   - ☐ Newly Executed Declaration (for CIP application)
   - ■ Deferred under 37 C.F.R. § 1.53(f)
   - ☐ Deletion of Inventor(s) - Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b)
   - ☐ Incorporation by Reference - The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein

3. ■ 4 Distinct sheets of ■ Formal ☐ Informal Drawings

4. ☐ Preliminary Amendment

5. ☐ Information Disclosure Statement
   - ☐ Form 1449
   - ☐ A copy of each cited prior art reference

6. ☐ Assignment
   - ☐ Assignment with Cover Sheet attached
   - ☐ Assignment filed in prior application. Application assigned to:

7. ■ Priority is hereby claimed under 35 U.S.C. § 119 based upon the following application(s):

Country	Application Number	Date of Filing (day, month, year)
U.S.	60/158,160	08 October 1999

8. ☐ Priority document(s)

9. ☐ Small Entity Statement
   - ☐ Small Entity Statement was filed in prior application, Small Entity Status is still proper and desired
   - ☐ is attached
   - ☐ is no longer claimed

10. ☐ Microfiche Computer Program (Appendix)

11. ☐ Nucleotide and/or Amino Acid Sequence Submission
   - ☐ Computer Readable Copy
   - ☐ Paper Copy (identical to computer copy)
   - ☐ Statement verifying identity of above copies

12. Calculation of Fees:

FEES FOR	EXCESS CLAIMS	FEE	AMOUNT DUE
Basic Filing Fee (37 C.F.R. § 1.16(a))			$690.00
Total Claims in Excess of 20 (37 C.F.R. § 1.16(c))	36	18.00	$648.00
Independent Claims in Excess of 3 (37 C.F.R. § 1.16(b))	2	78.00	$156.00
Multiple Dependent Claims (37 C.F.R. § 1.16(d))	0	260.00	$0.00
Subtotal - Filing Fee Due			$1,494.00
	MULTIPLY BY		
Reduction by 50%, if Small Entity (37 C.F.R. §§ 1.9, 1.27, 1.28)	0		$0.00
TOTAL FILING FEE DUE			$0.00
Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h))	0	40.00	$0.00
GRAND TOTAL DUE			**$1,494.00**

13. PAYMENT is:
   - ☐ included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18
   - ■ not included, but deferred under 37 C.F.R. § 1.53(f).

14. All correspondence for the attached application should be directed to:

Banner & Witcoff, Ltd.
1001 G Street, N.W.
Washington, D. C. 20001-4597
Telephone: (202) 508-9100
Facsimile: (202) 508-9299

15. Other: _____

Date: February 16, 2000

By: _____
Sarah A. Kagan
Reg. No. 32,141

SAK/ama

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

This invention was supported with U.S. government funds, NIH grants CA43460, CA57345, CA62924, CA67409, CA72851. The government therefore retains certain rights in the invention. This application claims the benefit of provisional application Serial No. 60/158,160 filed October 8, 1999, and is a continuation-in-part of patent application Serial No. 09/461,047 filed December 15, 1999.

BACKGROUND OF THE INVENTION

The problem with humans and other mammals, at least from a genetic diagnostic perspective, is that they are diploid. Mutations in one allele, such as those responsible for all dominantly inherited syndromes, are always accompanied by the wild-type sequence of the second allele. Though many powerful techniques for genetic diagnosis have been developed over the past decade, all are compromised by the presence of diploidy in the template. For example, the presence of a wild-type band of the same electrophoretic mobility as a mutant band can complicate interpretation of sequencing ladders, especially when the mutant band is of lower intensity. Deletions of a segment of DNA are even more problematic, as in such cases only the wild-type allele is amplified and analyzed by standard techniques. These issues present difficulties for the diagnosis of monogenic diseases and are even more problematic for multigenic diseases, where causative mutations can occur in any of several different genes. Such multigenism is the rule rather than the exception for common predisposition syndromes, such as those associated with breast and colon cancer, blindness, and hematologic, neurological, and cardiovascular diseases. The sensitivity of genetic diagnostics for these diseases is currently suboptimal, with 30% to 70% of cases refractory to genetic analysis.

There is a need in the art for simply separating and analyzing individual alleles from human and other mammalian cells.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for detecting mutations in a gene of interest on a human or other mammalian chromosome.

It is another object of the invention to provide a method for making test cells suitable for sensitive genetic testing.

It is yet another object of the invention to provide a population of fused cell hybrids which are useful for genetic analysis.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method of detecting mutations in a gene of interest of a human or other mammal is provided. Cells of a human or other mammal are fused to rodent cell recipients to form human-rodent or other mammal-rodent cell hybrids. Fused cell hybrids are selected by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human or other mammalian chromosome, forming a population of fused cell hybrids. A subset of hybrids are detected among the population of fused cell hybrids. The hybrids are haploid for a second human other mammalian chromosome which is not the same chromosome as the first human or other mammalian chromosome and which was not selected. The subset of hybrids are tested to detect a gene, an mRNA product of said gene, or a protein product of said gene. The gene resides on the second human or other mammalian chromosome. Diminished amounts of the mRNA or protein product or altered properties of the gene, mRNA, or protein product indicate the presence of a mutation in the gene in the human or other mammal.

According to another embodiment, a method is disclosed which provides test cells for genetic testing. The test cells are haploid for human or other mammalian genes. Cells of a human or other mammal are fused to transformed, diploid, rodent cell recipients to form human-rodent or other mammal-rodent cell hybrids. Fused cell hybrids are selected by selecting for a marker on each of a first human or other mammalian chromosome and a rodent chromosome, forming a population of cells

which stably main...n one or more human or other mamm... n chromosomes in the absence of selection for the human or other mammalian chromosomes. Cells which are haploid for a second human or other mammalian chromosome which is distinct from the first human or other mammlian chromosome are detected among the population of cells; the second human or other mammalian chromosome was not selected.

Also provided by the present invention is a population of rodent-human or rodent-other mammalian hybrid cells wherein each homolog of at least 2 human or other mammalian autosomes is present in haploid form in at least one out of one hundred of the cells.

The present invention thus provides the art with a method which can be used to increase the sensitivity and effectiveness of various diagnostic and analytic methods by providing hybrid cells to analyze which are haploid for one or more genes of interest. The human or other mammalian chromosome content of the hybrid cells is stable and uniform.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Strategy for hybrid generation. The recipient mouse cell line E2 was fused with human lymphocytes and clones were subsequently selected with HAT plus geneticin, which kill unfused E2 cells and lymphocytes, respectively. All clones contained a human X chromosome responsible for growth in HAT. Clones were genotyped to determine which human chromosomes were retained. Chromosomes marked "A" and "B" represent the two homologs of a representative human chromosome. The average proportion of clones which retained neither, both, or either of the six chromosome homologs analyzed is indicated (see text). Mutational analysis was carried out on nucleic acids of clones which retained single alleles of the genes to be tested.

Fig. 2. Allelic status and gene expression in hybrids. **(Fig. 2A)** Polymorphic markers from the indicated chromosomes were used to determine the genotype of the indicated hybrids. "Donor" denotes the human lymphocytes used for fusion with the mouse

recipient cells. (**Fig. 2B**) cDNA of E2 and four hybrids were used as templates to amplify *hMSH2, hMSH6, hMLH1, hTGF β-RII, hPMS1, hPMS2,* and *APC* sequences. The results were concordant with the genotypes observed in (**Fig. 2A**), in that hybrids 5 - 7 retained at least one allele of each of the chromosomes containing the tested genes, while hybrid 8 contained alleles of chromosomes 3, 5, and 7 but not of chromosome 2 (containing the *hMSH2, hPMS1, and hMSH6* genes).

Fig. 3. Mutational analysis of an HNPCC patient refractory to standard genetic diagnosis. Nucleic acids from the indicated hybrids were tested for retention of chromosomes 2 and 3 using polymorphic markers (**Fig. 3A**) and for expression of *hMSH2* and *hMLH1* genes on chromosomes 2 and 3, respectively (**Fig. 3B**). Hybrids 1, 2, 3, and 6 contained allele A from chromosome 2 and did not express hMSH2 transcripts, while hybrids 4 and 5 contained the B allele and expressed hMSH2. hMLH1 expression served as a control for the integrity of the cDNA. (**Fig. 3C**) Sequences representing the indicated exons of *hMSH2* were amplified from the indicated hybrids. Exons 1- 6 were not present in the hybrids containing allele A, but exons 7 - 16 were present in hybrids containing either allele.

Fig. 4. Mutational analysis of Warthin family G. (**Fig. 4A**) Sequence analysis of RT-PCR products from hMSH2 transcripts of hybrid 1, containing the mutant allele of a Warthin family G patient, illustrates a 24 bp insertion (underlined; antisense primer used for sequencing). The wild-type sequence was found in hybrid 3, containing the wt allele. RT-PCR analysis of transcripts from lymphoid cells of the patient showed that the mutant transcript was expressed at significantly lower levels than the wild-type sequence. Sequence analysis of the genomic DNA of the same hybrids (**Fig. 4B**) showed that the insertion was due to a A to C mutation (antisense sequence, indicated in bold and underlined) at the splice acceptor site of exon 4, resulting in the use of a cryptic splice site 24 bp upstream. The signal of the mutant C is not as strong as the wild-type A in the donor's DNA. Such non-equivalence is not unusual in sequencing templates from diploid cells, and can result in difficulties in interpretation of the

chromatograms. (Fig. 4C) Extracts from hybrids 1 and 5, carrying the mutant allele of chromosome 2, were devoid of hMSH2 protein, while extracts of hybrids 2 and 3, carrying the wt allele, contained hMSH2 protein. Hybrid 4 did not contain either allele of chromosome 2. Hybrids 1, 3, 4, and 5 each carried at least one allele of chromosome 3 and all synthesized hMLH1 protein. α-tubulin served as a protein loading control. Immunoblots with antibodies to the indicated proteins are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have devised a strategy for generating hybrids containing any desired human or other mammal's chromosome using a single fusion and selection condition. Importantly and unexpectedly, the human or other mamalian chromosomes in these hybrids were stable, and they expressed human or other mammalian genes at levels sufficient for detailed analysis. The approach is based on the principle that fusion between human or other mammal and rodent cells creates hybrid cells that contain the full rodent genomic complement but only a portion of the human or other mammalian chromosomes. In the past, selection for retention of a specific human or other mammalian chromosome (by complementation of an auxotrophic rodent cell, for example) has allowed the isolation of hybrids containing a desired chromosome (7, 8). Though such fusions have proven useful for a variety of purposes (8, 9), their utility has been limited by the availability of appropriate rodent recipients for many chromosomes and by the inefficiencies and variation of the fusion and selection conditions. For the analysis of multigenic diseases, it would be necessary to perform a separate fusion and selection for each chromosome.

The stability of the human or other mammalian chromosomes in the hybrids of the present invention was surprising. Though the human genetic constitution of radiation hybrids is relatively stable, this stability has been presumed to be due to the integration of small pieces of human DNA into rodent chromosomes following irradiation of the donor cells. The human chromosomes in whole cell fusions have been believed to be unstable unless continuous selection pressure for individual chromosomes was exerted. The reasons for the stability in our experiments is unclear, but may be related to the diploid nature of the rodent partner. Such diploidy reflects a chromosome

stability that is unusual among transformed rodent cells. Previous experiments have indeed shown that chromosomally stable human cells retain all chromosomes upon fusion with other chromosomally stable human cells, unlike the situation when one of the two partners is chromosomally unstable.

The diploid, rodent recipient cells of the present invention provide useful reagents for the facile creation of cells with functionally haploid human genomes. Nucleic acids or proteins from these hybrids can be used as reagents for any standard mutational assay. As mutational assays are constantly being improved and automated (1), the value of the hybrid-generated materials correspondingly increases. It may soon become possible, in fact, to examine the sequence of entire genes (promoters and introns in addition to exons). Nucleic acid templates generated from single alleles are clearly superior for such analyses, as the homogeneous nature of the templates dramatically enhances the signal to noise ratio of virtually any diagnostic assay. We therefore envision that this approach can be productively applied to a wide variety of research and clinical problems.

Genes of interest are typically those which have been found to be involved in inherited diseases. These include genes involved in colon cancer, breast cancer, Li-Fraumeni disease, cystic fibrosis, neurofibromatosis type 2, von Hippel-Lindau disease, as well as others. The identified genes include *APC*, *merlin*, *CF*, *VHL*, *hMSH2*, *p53*, *hPMS2*, *hMLH1*, *BRAC1*, as well as others. Mutations which can be identified at the protein level include those in sequences that regulate transcription or translation, nonsense mutations, splice site alterations, translocations, deletions, and insertions, or any other changes that result in substantial reduction of the full-length protein. Other subtler mutations can be detected at the nucleic acid level, such as by sequencing of RT-PCR products.

Cells of the human which may be used in fusions are any which can be readily fused to rodent cells. Peripheral blood lymphocytes (PBL) which are readily available clinical specimens are good fusion partners, with or without prior mitogenetic stimulation, whether used fresh or stored for over one year at -80° C. Since inherited mutations are the subject of the present method, any cells of the human body can be used, since all such cells contain essentially the same genetic

complement. Cells of other mammals which can be used include in particular those of cats, dogs, cows, sheep, goats, horses, chimpanzees, baboons, and hogs. More generically, the cells of the other mammals can be selected from the ruminants, primates, carnivora, lagomorpha, and perissodactyla. Typically the other mammalian cell fusion partner is not a rodent cell.

Rodent cell recipients for fusion are preferably diploid, more preferably oncogene-transformed, and even more preferably have microsatellite instability due to a defect in a mismatch repair gene. Selection of particular clones which grow robustly, are stably diploid, and fuse at a high rate is well within the skill of the ordinary artisan. The rodent cells may be, for example, from mice, rats, guinea pigs, or hamsters.

Fusion of cells according to the present invention can be accomplished according to any means known in the art. Known techniques for inducing fusion include polyethylene glycol-mediated fusion, Sendai virus-mediated fusion, and electro-fusion. Cells can desirably be mixed at a ratio of between 10:1 and 1:10 human to rodent. Clones of fused cells generally become visible after about two to three weeks of growth.

Fused hybrid cells can be selected using any markers which result in a positively selectable phenotype. These include antibiotic resistance genes, toxic metabolite resistance genes, prototrophic markers, etc. The surprising advantage of the present invention is that a single marker on a single human or other mammalian chromosome can be used in the selection, and that stable hybrids containing more than just the single, selected human or other mammalian chromosome result. Thus markers on other chromosomes can be analyzed even when the chromosomes on which the markers reside were not selected.

Fused hybrid cells can be analyzed to determine that they do in fact carry a human or other mammalian (non-rodent) chromosome which carries a gene of interest. Hybrid cells which have either of the two relevant human or other mammalian chromosomes can be distinguished from each other as well as from hybrids which contain both of the two human or other mammalian chromosomes.

See **Fig. 1.** While any means known in the art for identifying the human or other mammalian chromosomes can be used, a facile analysis can be performed by assessing microsatellite markers on the human or other mammalian chromosome. Other linked polymorphic markers can be used to identify a desired human or other mammalian chromosome in the hybrids.

Once hybrid cells are isolated which contain one copy of a human or other mammalian gene of interest from a human or other mammal who is being tested, mutation analysis can be performed on the hybrid cells. The genes can be tested directly for mutations, or alternatively the mRNA or protein products of the genes can be tested. Mutations that result in reduced expression of the full-length gene product should be detectable by Western blotting using appropriate antibodies. Tests which rely on the function of the protein encoded by the gene of interest and enzyme assays can also be performed to detect mutations. Other immunological techniques can also be employed, as are known in the art.

If an immunological method is used to detect the protein product of the gene of interest in the hybrids, it is desirable that antibodies be used that do not cross-react with rodent proteins. Alternatively, the rodent genes which are homologous to the gene of interest can be inactivated by mutation to simplify the analysis of protein products. Such mutations can be achieved by targeted mutagenesis methods, as is well known in the art.

Functional tests can also be used to assess the normalcy of each allelic product. For example, if one inserted an expression construct comprising a β-galactosidase gene downstream from a p53 transcriptional activation site, into a rodent-human hybrid cell that contained human chromosome 17 but no endogenous p53, then one could detect mutations of the p53 on the human chromosome 17 by staining clones with X-gal. Other enzymatic or functional assays can be designed specifically tailored to the gene of interest.

Any method of detecting mutations at the DNA or RNA level as are known in the art may be employed. These include without limitation, sequencing, allele-

specific PCR, allele-specific hybridization, microarrays, DGGE, and automated sequencing.

It is a possibility that expression of the gene of interest might be inhibited in the hybrid cell environment. In order for the loss of expression of a gene of interest in the hybrid cells to be meaningfully interpreted as indicating a mutation in the human or other mammal, one must confirm that the gene of interest, when wild-type, is expressed in rodent-human or other mammal hybrid cells. This confirmation need not be done for each patient, but can be done once when the assay is being established.

When the assay of the present invention indicates that a mutation exists in the gene of interest, other family members can be tested to ascertain whether they too carry the mutation. Alternatively, the other family members can be tested to see if they carry the same chromosome as the affected family member. This can be determined by testing for a haplotype, *i.e.*, a set of distinctive markers which are found on the chromosome carrying the mutation in the affected family member. Determination of a haplotype is a by-product of performing the assay of the invention on the first family member. When the hybrid cells are tested to confirm the presence of the relevant chromosome in the hybrid, for example by use of microsatellite markers, a distinctive marker set will be identified, which can then be used as a haplotype.

Mixed populations of hybrid cells made by the fusion process of the present invention may contain hybrid cells which are haploid for a number of different human or other mammalian chromosomes. Typically each homolog of at least 2, at least 5, at least 10, at least 15, at least 20, or even 22 human or other mammalian autosomes will be present in the population in a haploid condition in at least one out of one hundred, seventy-five, fifty, thirty or twenty-eight of the cells. Thus a high proportion of the cells contain multiple human or other mammalian chromosomes, and a relatively small number of cells must be tested to find cells harboring a single copy of a non-selected chromosome.

Populations of cells resulting from a single hybrid are uniform and homogeneous due to the high stability of the human or other mammalian chromosomes in the hybrid cells of the invention. Thus at least 75%, 80%, 85%, 90%, 95%, 97%, 99%, or 100% of the cells in the population resulting from a single hybrid cell contain the same complement of human or other mammalian chromosomes.

The following examples provide experimental details which demonstrate one of many ways to carry out the invention. The invention is not limited to the particular methods of cells employed in the examples. The claims and the specification as a whole provide the measure of the invention.

Examples

Example 1

An outline of the approach is presented in Fig. 1. The rodent fusion partner was a line derived from mouse embryonic fibroblasts transformed with ras and adenovirus E1A oncogenes. HPRT-deficient subclones of this line were generated, and one subclone (E2) was chosen for further experimentation based on its robust growth characteristics, maintenance of diploidy, and fusion efficiency (10). Human lymphocytes cells were mixed with E2 cells at an optimum ratio and electrofused, and hybrids selected in geneticin (to kill unfused human cells) and HAT (to kill unfused E2 cells) (11). Colonies appearing after two weeks of growth were expanded and RNA and DNA prepared for analysis. From a single fusion experiment, an average of 36 hybrid clones were obtained (range of 17 to 80 in five different individuals).

All hybrids contained the human X chromosome, as this chromosome contains the HPRT gene allowing growth in HAT. To determine whether other human chromosomes were present in the hybrids, polymorphic microsatellite markers (12) were used as probes in PCR-based assays (Fig. 2A). We focused on the chromosome arms (2p, 2q, 3p, 5q, 7q, and 16q) known to contain colorectal cancer (CRC) predisposition genes. One copy of each of these chromosome arms was present in a significant fraction of the hybrid clones. For example, of 476 hybrids derived from 14 individuals and examined for chromosome 3, 136 hybrids contained neither donor chromosome, 211 hybrids contained both donor chromosomes, 60 hybrids contained one parent's

chromosome, and 39 hybrids contained the other parental chromosome. Similar retention frequencies were found for all six chromosome arms analyzed. Testing of markers from both arms of a single chromosome showed that whole chromosomes, rather than chromosome fragments, were generally retained in the hybrids. This result was confirmed with fluorescence in situ hybridization (FISH) on metaphase spreads from the hybrids, which indicated the presence of 11 ±3 human chromosomes in each hybrid cell. Calculations based on the genotypic data indicated that the analysis of 25 hybrids would ensure a 95% probability of identifying at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of a single chromosome under study. Moreover, it would require only 45 hybrids to similarly ensure that each allele of all 22 autosomes was present and separated from its homolog in at least one hybrid (13).

Example 2

Two other features of the hybrids were essential for the analyses described below. First, the human chromosome complements of the hybrids were remarkably stable. Polymorphic marker analysis in ten hybrids revealed identical patterns of retention after growth for 90 (30 passages) generations after initial genotyping. Second, those hybrids containing the relevant chromosome expressed every human gene assessed, including all known colorectal cancer susceptibility genes (the *hMSH2* and *hMSH6* genes on chromosome 2p, the *hPMS1* gene on chromosome 2q, the *TGF- β Receptor Type II* gene and *hMLH1* gene on chromosome 3p, the *APC* gene on chromosome 5q, the *hPMS2* gene on chromosome 7q, and the *E-cadherin* gene on chromosome 16q; representative examples in Fig. 2B) (14).

Example 3

Having established the stability and expression patterns of CRC-predisposition genes in these hybrids, we used this "conversion" approach to investigate ten patients who had proven refractory to standard genetic diagnostic techniques. Each of these patients had a significant family history of colorectal cancer and evidence of mismatch repair deficiency in their tumors, yet sequencing of the entire coding sequence of each known MMR gene had failed to reveal mutations. Indeed, these and similar studies have

prompted the speculation that other major HNPCC genes must exist. (25-34) Hybrids were generated from lymphocytes of each patient, and at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of each MMR gene was isolated. Analysis of the nucleic acids from these hybrids revealed specific mutations in all ten patients (Table 1). In every case, an abnormality was found in a single allele of either *hMSH2* or *hMLH1*. The nature of the abnormalities revealed why they had not been detected with the standard methods previously used for their analysis. Three cases were due to large deletions, encompassing six or seven exons. When corresponding nucleic acids from the cells of such patients are evaluated by any PCR-based method, only the wild type sequences from the unaffected parent would be amplified, leading to the false impression of normalcy (for example, case #1 in Figure 3). Though Southern blotting can reveal deletions of one or a few exons in MMR, larger deletions are refractory to such blotting methods. In three cases (#4, 6, and 9), no transcript was generated from one allele, though the sequences of all exons and intron-exon borders from this allele were normal. Presumably, mutations deep within an intron or within the promoter of the gene were responsible. The absence of transcripts from one specific allele of these three patients was confirmed in at least three other converted hybrids from each patient. In four other cases, point mutations were found (Table 1). These mutations were not detected in the original sequence analyses because the signals from the mutant allele were not as robust as those from the wild type. Such asymmetry can be caused by instability of mutant transcripts due to nonsense mediated decay (36-38), or to nucleotide preferences of the polymerases in specific sequence contexts, and represents a common problem for both manual and automated sequencing methods (39). The conversion approach eliminates these problems because only one sequence can possibly be present at each position. A good example of this was provided by Warthin G (17). The mutation in this prototype kindred was an A to C transversion at a splice site. The signal from the mutant "C" in the sequencing ladder was not as intense as the wild type "A" (Fig. 4b). This mutation led to the use of a cryptic splice site 24 bp upstream of exon 4, and an under-represented transcript with a 24 base insertion (Fig. 4a). To demonstrate that this mutation had an effect at the protein level, we analyzed the hybrids by immunoblotting with specific antibodies (19). The hybrids containing the

mutant allele did not make detectable levels of human hMSH2 protein, though they did synthesize normal levels of a control protein (Fig. 4C).

The results described above demonstrate that individual alleles of human chromosomes can be readily isolated upon fusion to mouse cells.

HNPCC provides a cogent demonstration of the power of the conversion approach because it is a common genetic disease that has been widely studied. In the last three years, for example, extensive analyses of the major MMR genes have been performed in 303 HNPCC kindreds from nine cohorts distributed throughout the world (25-34). Based on the fraction of such patients with characteristic microsatellite instability in their cancers (30-34), it can be estimated that 239 (78%) of the kindreds had germ-line mutations of mismatch repair genes. Yet MMR gene mutations were identified in only 127 (42%) of these 239 kindreds (25-34). Our cohort was similar, in that it was derived from a total of 25 kindreds, 22 of whom had tumors with microsatellite instability and presumptive MMR gene mutations. Of these 22, our initial analyses revealed mutations in only 12 (54%) (ref. 14 and unpublished data). Mutations of the other ten patients were only revealed upon conversion analysis, which thereby increased the sensitivity from 54% to 100%. The conclusion that virtually all cases of HNPCC associated with MSI are due to germline mutations of known MMR genes is consistent with recent immunohistochemical data demonstrating the absence of either MSH2 or MLH1 protein staining in the cancers from the great majority of HNPCC patients (40, 41). A corollary of these results is that the search for new human MMR genes should not based on the premise that a large fraction of HNPCC cases will prove attributable to such unknown genes.

The system described above can be applied to other genetic diseases in a straight forward manner. It should be emphasized that this approach is not a substitute for the many powerful methods currently available to search for specific mutations. Rather, conversion can be used to maximize the sensitivity of existing techniques. Converted nucleic acids provide the preferred substrates for such methods because of the higher signal to noise attainable and the inability of the wild type allele to mask or confound detection of the mutant allele. As DNA-based mutational assays are improved in the future, and progressive incorporate microarrays and other automatable features (42-44),

the value of conversion-generated nucleic acids will correspondingly increase, significantly enhancing the effectiveness of genetic tests for hereditary disease.

Methods

Cell culture

Mouse embryonic fibroblasts were derived from MSH2-deficient mice (46) and transformed with adenovirus E1A and RAS oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

Cell fusion and the generation of hybrids

The patients were from kindreds with HNPCC as defined by the Amsterdam criteria (44); in no case was linkage analysis feasible due to the lack of a sufficient number of affected individuals. Microsatellite instability (MSI) in the cancers from these patients was determined through the markers recommended in ref. 45. 3 x 10^6 E2 cells and 12 x 10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX ElectroCell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersburg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. From a single fusion, an average of 23 +/- 15 hybrid clones were obtained. The lymphocytes used for the experiments described here were derived from Epstein-Barr Virus infection of peripheral blood leukocytes, but it was found that freshly drawn lymphocytes could also be successfully fused and analyzed using identical methods.

Genotyping

Genotyping was performed as described (12). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788 and D2S1360, D2S1384, D3S2406, D7S1824, and D16S3095, from chromosome 2p, 2q, 3p, 5q, 7q and 16q, respectively. Fluorescence in situ hybridization was performed as described previously (21).

PCR and sequencing

Polyadenylated RNA was purified and RT-PCR performed as described previously. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through an internet site.

Statistical analysis

the number of hybrids containing none, both or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the number of hybrids required to generate mono-allelic hybrids containing specific numbers of each chromosomes.

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10. E2 cells were derived from mouse embryonic fibroblasts derived from MSH2-deficient mice (generously provided by T. Mak) and transformed with adenovirus *E1A* and *RAS* oncogenes. HPRT-deficient subclones were selected

[1]All references are explicitly incorporated by reference.

by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

11. 3×10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX Electro Cell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersberg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. The lymphocytes used for the experiments described here were derived from Epstein-Barr virus infection of peripheral blood leukocytes, but we found that freshly drawn lymphocytes could also be successfuly fused and analyzed using identical methods

12. Genotyping was performed as described in F.S. Leach et al., *Cell* 75, 1215 (1993). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788, D2S13360, D3S2406, D7S1824, and D16S3095, from chromosomes 2p, 2q, 3p, 5q, and 16q, respectively.

13. The numbers of hybrids containing none, both, or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the numbers of hybrids required to generate mono-allelic hybrids containing specific numbers of chromosomes.

14. Polyadenylated RNA was purified and RT-PCR performed as described in B. Liu et al., *Nat Medicine* **2**, 169 (1996).

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18. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through a *Science* internet site.

19. Cytoplasmic extracts of cells hybrids were separated by electrophoresis through SDS-polyacrylamide gels and immunoblotted with antibodies specific for human hMSH2 (#NA26, Calboiochem), human hMLH1 (#13271A, Pharmingen), or β-Tubulin (#N357, Amersham).

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CLAIMS

1. A method of detecting mutations in a gene of interest of a non-rodent mammal, comprising the steps of:

fusing cells of a non-rodent mammal to rodent cell recipients to form non-rodent mammal-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first non-rodent mammalian chromosome, to form a population of fused cell hybrids;

detecting among the population of fused cell hybrids a subset of hybrids which are haploid for a second non-rodent mammalian chromosome which is not the same chromosome as the first non-rodent mammalian chromosome and which was not selected;

analyzing said subset of hybrids to detect a mutation in an mRNA product of a gene or in said gene, wherein the gene resides on the second non-rodent mammalian chromosome.

2. The method of claim 1 wherein the rodent cell is diploid.

3. The method of claim 1 wherein the rodent cell recipients are deficient in mismatch repair.

4. The method of claim 1 wherein the rodent cell recipient is geneticin resistant.

5. The method of claim 1 wherein the rodent cell recipients are transformed with an oncogene.

6. The method of claim 5 wherein the oncogene is *ras*.

7. The method of claim 5 wherein the oncogene is *E1A*.

8. The method of claim 1 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

9. The method of claim 1 wherein the rodent cell recipients are *MSH2*$^-$.

10. The method of claim 1 wherein the rodent cell recipient is diploid, and contains both a selectable marker and a counterselectable marker.

11. The method of claim 10 wherein the counterselectable marker is HPRT deficiency.

12. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third non-rodent mammalian chromosome, wherein the first, second, and third non-rodent mammalian chromosomes are distinct, and neither the second nor the third non-rodent mammal chromsomes were selected.

13. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third and fourth non-rodent mammalian chromosome, wherein the first through fourth non-rodent mammalian chromosomes are distinct, and neither the second through fourth non-rodent mammal chromsomes were selected.

14. The method of claim 12 further comprising the step of testing said subset of hybrids to detect an mRNA product of a second gene or a protein product of said second gene, wherein the second gene resides on the third non-rodent mammalian chromosome, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product from said second gene indicate the presence of a mutation in the second gene in the non-rodent mammal.

15. The method of claim 13 testing said subset of hybrids to detect an mRNA product of a second and third gene or a protein product of said second and third gene, wherein the second and third genes reside on the third and fourth non-rodent mammalian chromosomes, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product of said third or fourth genes indicate the presence of a mutation in third or fourth genes in the non-rodent mammal.

16. The method of claim 1 wherein the cells of the non-rodent mammal are lymphocytes.

17. The method of claim 1 wherein the step of detecting a subset of hybrids which are haploid for the second non-rodent mammalian chromosome is

accomplished by identifying the presence of a microsatellite marker on the second non-rodent mammalian chromosome.

18. The method of claim 1 wherein the step of analyzing to detect a mutation is performed by a technique selected from the group consisting of: nucleic acid sequencing, allele-specific PCR, allele-specific hybridization, hybridization to a microarray, discontinuous gradient gel electrohoresis (DGGE), and automated nucleic acid sequencing.

19. A method of providing test cells for genetic testing, wherein said test cells are haploid for genes of a non-rodent mammal, comprising the steps of:

fusing cells of a non-rodent mammal to transformed, diploid, rodent cell recipients to form non-rodent mammal-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a marker on each of a first non-rodent mammalian chromosome and a rodent chromosome, whereby a population of cells are formed which stably maintain one or more non-rodent mammalian chromosomes in the absence of selection for the non-rodent mammalian chromosomes;

detecting among the population of cells those cells which are haploid for a second and third non-rodent mammalian chromosome which are distinct from the first non-rodent mammalian chromosome and which were not selected.

20. The method of claim 19 wherein the rodent cell recipient is geneticin resistant.

21. The method of claim 19 wherein the rodent cell recipients are transformed with an oncogene.

22. The method of claim 21 wherein the oncogene is *ras*.

23. The method of claim 21 wherein the oncogene is *E1A*.

24. The method of claim 19 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

25. The method of claim 19 wherein the rodent cell recipients are deficient in mismatch repair.

26. The method of claim 19 wherein the rodent cell recipient contains a counterselectable marker.

27. The method of claim 26 wherein the counterselectable marker is HPRT deficiency.

28. The method of claim 19 wherein the cells of the non-rodent mammal are lymphocytes.

29. The method of claim 19 wherein the step of detecting cells which are haploid for said second and third non-rodent mammalian chromosomes is performed by identifying the presence of a microsatellite marker on said second and third non-rodent mammalian chromosomes.

30. The method of claim 19 further comprising the step of:

detecting among the population of cells, cells haploid for a fourth non-rodent mammalian chromosome which is distinct from the first, second, and third non-rodent mammalian chromosomes and which was not selected.

31. The method of claim 19 futher comprising the step of:

detecting among the population of cells, cells haploid for a fourth, fifth, and sixth non-rodent mammalian chromosome, wherein said fourth through sixth non-rodent mammalian chromosomes are distinct from the first, second and third non-rodent mammalian chromosomes and were not selected.

32. The method of claim 19 further comprising:

testing nucleic acids of a cell haploid for the third non-rodent mammalian chromosome for a mutation in a gene on the third non-rodent mammalian chromosome.

33. The method of claim 19 further comprising:

testing proteins of a cell haploid for the second and third non-rodent mammalian chromosome for a mutation in a gene on each of the second and third non-rodent mammalian chromosomes.

34. The method of claim 1 wherein mRNA of a cell in the subset of hybrids is tested for a mutation in a gene on the second non-rodent mammalian chromosome.

35. The method of claim 1 wherein proteins of a cell in the subset of hybrids are tested for a mutation in a gene on the second non-rodent mammalian chromosome.

36. A population of rodent-non-rodent mammal hybrid cells wherein each homolog of at least 2 non-rodent mammal autosomes is present in haploid form in at least one out of one hundred of the cells.

37. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

38. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

39. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

40. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

41. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

42. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of a thirty of the cells.

43. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

44. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

45. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

46. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

47. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

48. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

49. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

50. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

51. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

52. A population of rodent-non-rodent mammal hybrid cells which stably maintain their non-rodent mammalian chromosome content such that at least 95% of the hybrid cells contain the same non-rodent mammalian chromosomes.

53. The population of rodent-non-rodent mammal hybrid cells of claim 52 whererein at least 97% of the hybrid cells contain the same non-rodent mammalian chromosomes.

54. The population of rodent-non-rodent mammal hybrid cells of claim 52 whererein at least 99% of the hybrid cells contain the same non-rodent mammalian chromosomes.

55. The method of claim 1 wherein the non-rodent mammal is a human.

56. The method of claim 1 wherein the non-rodent mammal is selected from the group consisting of goats, sheep, horse, cows, pigs, hogs, cats, and dogs.

ABSTRACT OF THE DISCLOSURE

Detection of mutations associated with hereditary diseases is complicated by the diploid nature of mammalian cells. Mutations present in one allele are often masked by the wild-type sequence of the other allele. Individual alleles can be isolated from every chromosome within somatic cell hybrids generated from a single fusion. Nucleic acids from the hybrids can be analyzed for mutations in an unambiguous manner. This approach was used to detect two cancer-causing mutations that had previously defied genetic diagnosis. One of the families studied, Warthin Family G, was the first kindred with a hereditary colon cancer syndrome described in the biomedical literature.

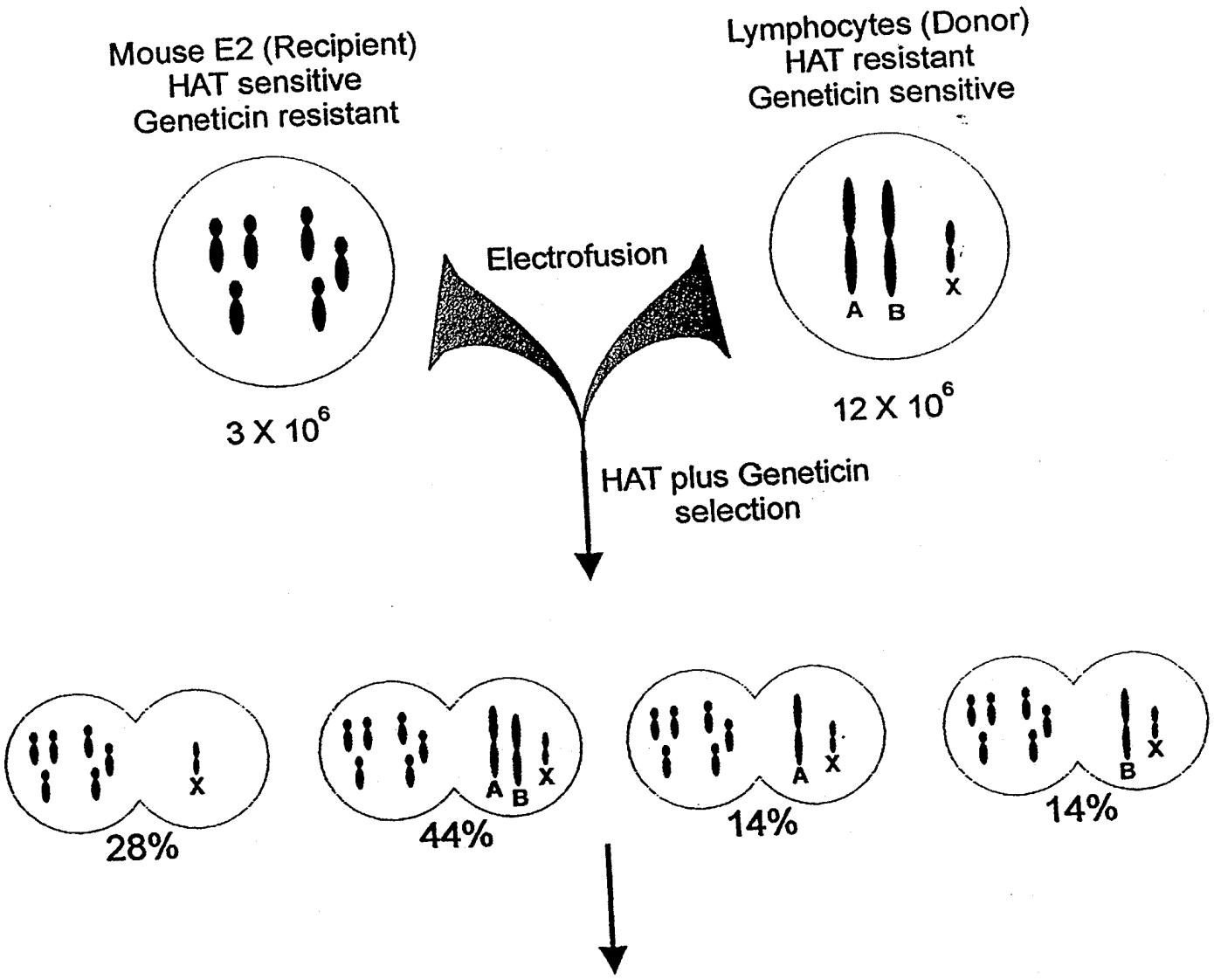

Figure 1
Yan *et al.*

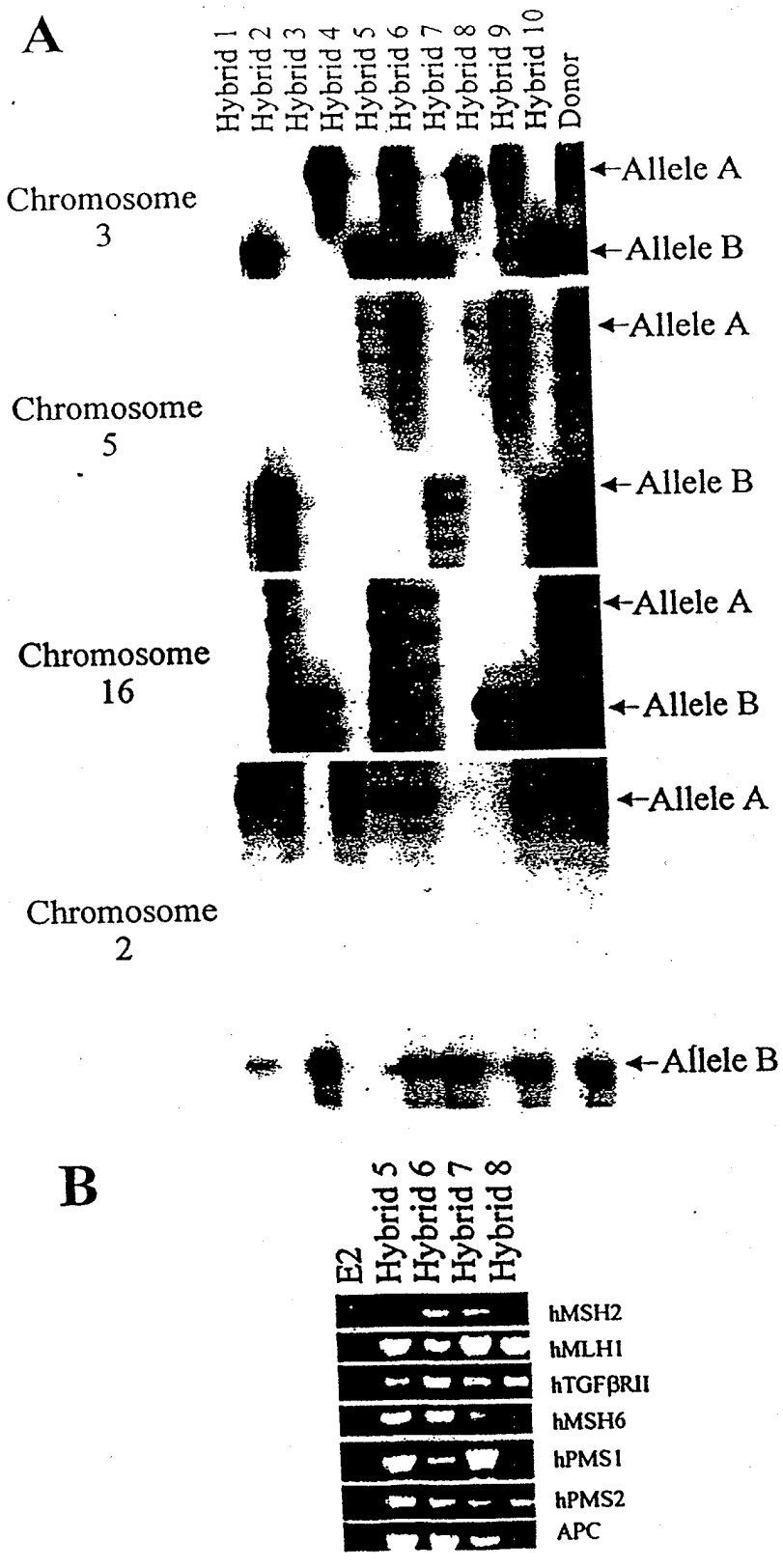

Figure 2
Yan *et al.*

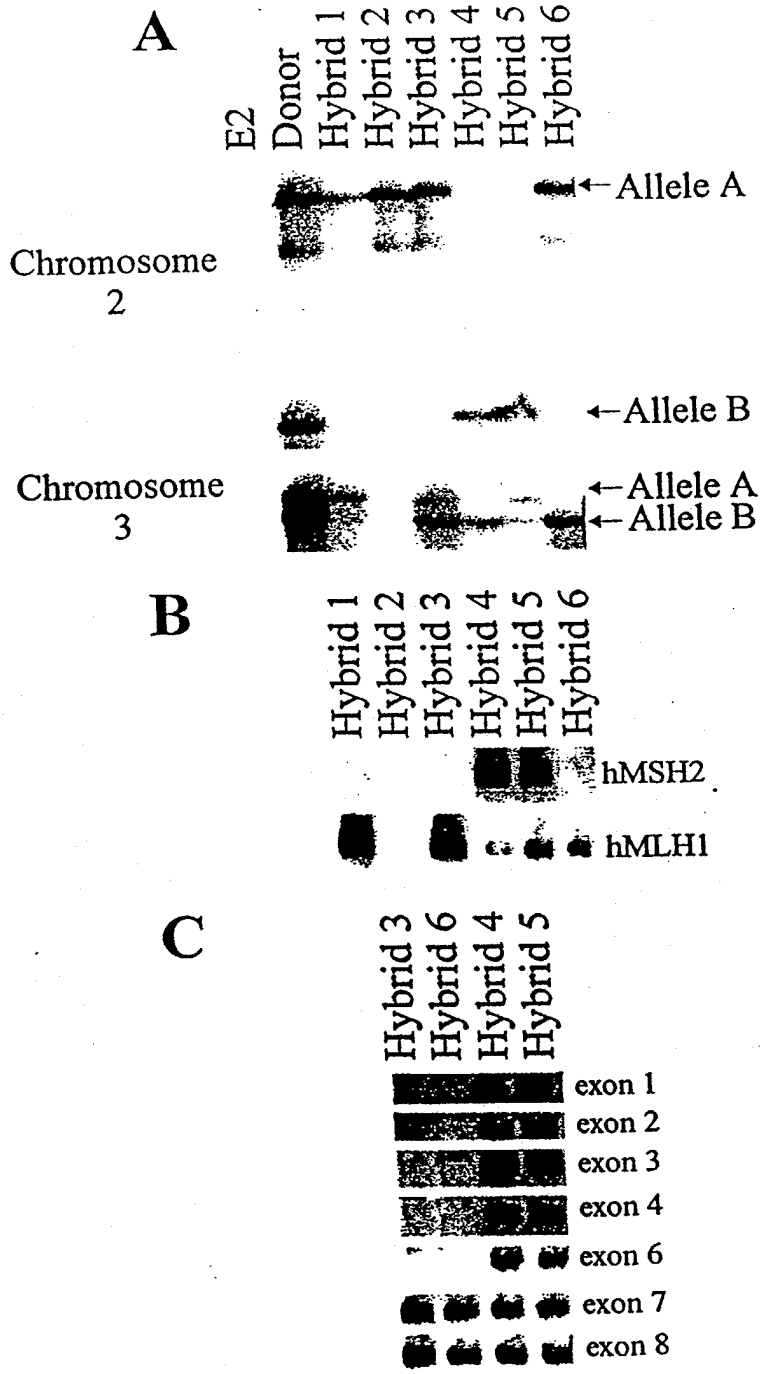

Figure 3
Yan *et al.*

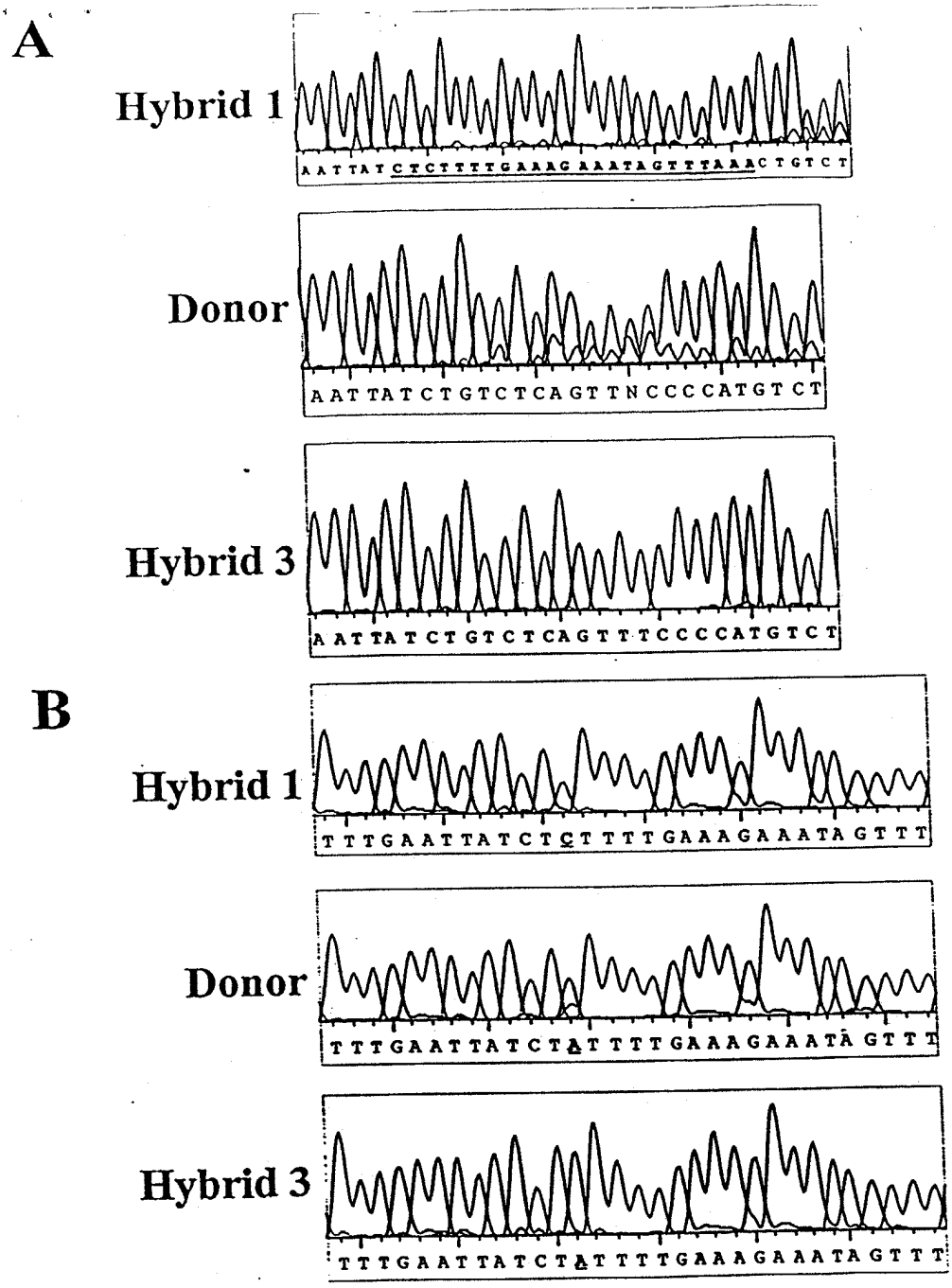

Figure 4
Yan *et al.*

PATENT COOPERATION TREATY

#5

From the RECEIVING OFFICE

PCT

To:

SARAH A. KAGAN
BANNER & WITCOFF, LTD.
1001 G STREET, N.W.
ELEVENTH FLOOR
WASHINGTON DC 20001-4597

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing (*day/month/year*)	27 OCT 2000
Applicant's or agent's file reference: 01107.00054	**IMPORTANT NOTIFICATION**

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/US00/27508	06 OCT 00	08 OCT 99

Applicant: THE JOHNS HOPKINS UNIVERSITY

Title of the invention: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:

☒ was transmitted to the International Bureau on 27 OCT 2000.

☐ has not yet been transmitted to the International Bureau for the reason indicated below and a copy of this notification has been sent to the International Bureau*:

☐ because the necessary national security clearance has not yet been obtained.

☐ because (*reason to be specified*):

* The International Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c)).

3. FOREIGN TRANSMITTAL LICENSE INFORMATION — Completed by: D. Jones

☑ Additional license for foreign transmittal not required. This subject matter is covered by a license already granted on the equivalent U.S. national application. Refer to that license for information concerning its scope.

☐ License for foreign transmittal not required. 37 CFR 5.11(e)(1) or 37 CFR 5.11(e)(2). However, a license may be required for additional subject matter. See 37 CFR 5.15(b).

☐ Foreign transmittal license granted. 35 U.S.C. 184; 37 CFR 5.11 on 10-14-00 (date):

☐ 37 CFR 5.15(a) ☐ 37 CFR 5.15(b)

Name and mailing address of the receiving Office Assistant Commissioner for Patents Box PCT Washington, D.C. 20231 Attn: RO/US	Authorized officer Virginia Irby PCT Operations - IAPD Team 1
Facsimile No.	Telephone No. (703) 305-3749 (703) 305-3230 (FAX)

Form PCT/RO/105 (July 1992)

PCT/US00/27508

PATENT COOPERATION TREATY

+ Demand due 5-8-01

PCT

From the INTERNATIONAL BUREAU

To:

KAGAN, Sarah, A.
Banner & Witcoff, Ltd.
Eleventh floor
1001 G Street, N.W.
Washington, DC 20001-4597
ETATS-UNIS D'AMERIQUE

NOTIFICATION OF RECEIPT OF RECORD COPY

RECEIVED (PCT Rule 24.2(a))

NOV 29 2000

BANNER & WITCOFF LTD.

MH 12/5/00

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
13 November 2000 (13.11.00)	
Applicant's or agent's file reference	**International application No.**
01107.00054	PCT/US00/27508

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

THE JOHNS HOPKINS UNIVERSITY (for all designated States except US)
VOGELSTEIN, Bert et al (for US)

International filing date	:	06 October 2000 (06.10.00)
Priority date(s) claimed	:	08 October 1999 (08.10.99) 15 December 1999 (15.12.99) 16 February 2000 (16.02.00)
Date of receipt of the record copy by the International Bureau	:	31 October 2000 (31.10.00)
List of designated Offices	:	

AP :GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW
EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
National :AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EE, ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA, MD,MG,MK,MN,MW,MX,MZ,NO,NZ,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US, UZ,VN,YU,ZA,ZW

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740 14 35

Authorized officer:

Catherine Massetti

Telephone No. (41-22) 338 83 38

Continuation of Form PCT/IB/301

NOTIFICATION OF RECEIPT OF RECORD COPY

Date of mailing (day/month/year) 13 November 2000 (13.11.00)	**IMPORTANT NOTIFICATION**
Applicant's or agent's file reference 01107.00054	**International application No.** PCT/US00/27508

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- [X] time limits for entry into the national phase
- [ ] confirmation of precautionary designations
- [X] requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

ANNEX TO FORM PCT/IB/301

national application No.
PCT/US00/27508

INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated in the Notification of Receipt of Record Copy (Form PCT/IB/301) by paying national fees and furnishing translations, as prescribed by the applicable national laws.

The time limit for performing these procedural acts is **20 MONTHS** from the priority date or, for those designated States which the applicant elects in a demand for international preliminary examination or in a later election, **30 MONTHS** from the priority date, provided that the election is made before the expiration of 19 months from the priority date. Some designated (or elected) Offices have fixed time limits which expire even later than 20 or 30 months from the priority date. In other Offices an extension of time or grace period, in some cases upon payment of an additional fee, is available.

In addition to these procedural acts, the applicant may also have to comply with other special requirements applicable in certain Offices. **It is the applicant's responsibility** to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most designated Offices do not issue reminders to applicants in connection with the entry into the national phase.

For detailed information about the procedural acts to be performed to enter the national phase before each designated Office, the applicable time limits and possible extensions of time or grace periods, and any other requirements, see the relevant Chapters of Volume II of the PCT Applicant's Guide. Information about the requirements for filing a demand for international preliminary examination is set out in Chapter IX of Volume I of the PCT Applicant's Guide.

GR and ES became bound by PCT Chapter II on 7 September 1996 and 6 September 1997, respectively, and may, therefore, be elected in a demand or a later election filed on or after 7 September 1996 and 6 September 1997, respectively, regardless of the filing date of the international application. (See second paragraph above.)

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date. If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with an indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity to furnish the priority document within a time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PCT

RO/US BOX PCT

Inspected By: AC

B&W # 01107.00054 Atty/Sec SAK/MH Date OCTOBER 6, 2000

In re International Application of THE JOHNS HOPKINS UNIVERSITY

International Application No. TBA

For CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS #9

The following has been received in the U.S. Receiving Office on the date stamped hereon:

- [X] New PCT Application & Fee, including:
- [ ] Resp. to Invitation to Correct Defects including:
- [ ] Chapter II Demand & Fee, including:
  # of sheets in Demand _____
- [ ] Article 19 Amendment, including:
- [ ] Response to Written Opinion, including:

OTHER ITEMS, included:

- [ ] _____
- [ ] _____
- [ ] _____
- [ ] _____
- [ ] _____
- [ ] _____

- [X] Transmittal Letter: 1 Sheet
- [X] Request: # of Sheet(s) 5
  Replacement Sheet(s) [ ] YES [X] NO
- [X] Fee Calculation Sheet: Original + 1 copy
  Description: # of Sheet(s) 18
  Replacement Sheet(s) [ ] YES [X] NO
- [X] Claims: # of Sheet(s) 6
  Replacement Sheet(s) [ ] YES [X] NO
- [X] Abstract: 1 Sheet
  Replacement Sheet [ ] YES [X] NO
- [ ] Formal Drawings: # of Sheet(s) _____
- [X] Informal Drawings: # of Sheet(s) 4
- [ ] Sequence Listing: # of Sheet(s) _____
- [ ] Executed Power(s) of Attorney: # of POA(s) _____
- [X] Check # 155939 for $ 2413.00

B&W Rev. 4/00

TRANSMITTAL LETTER TO THE UNITED STATES RECEIVING OFFICE

Date	6 October 2000
International Application No.	TBA
Attorney Docket No.	01107.00054

I. Certification under 37 CFR 1.10 (if applicable)

Express Mail mailing number	Date of Deposit

I hereby certify that the application/correspondence attached hereto is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231

Signature of person mailing correspondence	Typed or printed name of person mailing correspondence

II. [X] New International Application

TITLE	CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS	Earliest priority date (Day/Month/Year): 08 October 1999 (08.10.99)

SCREENING DISCLOSURE INFORMATION: In order to assist in screening the accompanying international application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied. (Note: check as many boxes as apply):

A. [ ] The invention disclosed was not made in the United States.
B. [ ] There is no prior U.S. application relating to this invention.
C. [X] The following prior U.S. application(s) contain subject matter which is related to the invention disclosed in the attached international application. (NOTE: *priority to these applications may or may not be claimed on form PCT/RO/101 (Request) and this listing does not constitute a claim for priority)*

application no.	60/158,160	filed on	08 October 1999 (08.10.99)
application no.	09/461,047	filed on	15 December 1999 (15.12.99)
application no.	09/504,860	filed on	16 February 2000 (16.02.00)

D. [X] The present international application [**X**] is identical to [ ] contains less subject matter than that found in the prior U.S. application(s) identified in paragraph C above.
E. [ ] The present international application [ ] contains additional subject matter not found in the prior U.S. application(s) identified in paragraph C above. The additional subject matter [X] **DOES NOT ALTER** [ ] **MIGHT BE CONSIDERED TO ALTER** the general nature of the invention in a manner which would require the U.S. application to have been made available for inspection by the appropriate defense agencies under 35 U.S.C. 181 and 37 CFR 5.1. See 37 CFR 5.15.

III. [ ] A Response to an Invitation from the RO/US. The following document(s) is(are) enclosed:

A. [ ] A Request for An Extension of Time to File a Response
B. [ ] A Power of Attorney (General or Regular)
C. [ ] Replacement pages:

pages		of the request (PCT/RO/101)	pages		of the figures
pages		of the description	pages		of the abstract
pages		of the claims			

D. [ ] Submission of Priority Documents
E. [ ] Fees as specified on attached Fee Calculation sheet form PCT/RO/101 annex

IV. [ ] A Request for Rectification under PCT Rule 91 [ ] A Petition [ ] A Sequence Listing Diskette

V. [ ] Other (please identify):

The person signing this form is the: ☐ Applicant ■ Attorney/Agent (Reg. No. 32,141) ☐ Common Representative

Typed name of signer	Sarah A. Kagan	Signature	

For receiving Office use only

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) 01107.00054

Box No. I TITLE OF INVENTION
CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

THE JOHNS HOPKINS UNIVERSITY
111 Market Place, Suite 906
Baltimore, Maryland 21202
United States of America

[ ] This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No. N/A

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
[ ] all designated States
[ ] the United States of America only
[X] all designated States except the United States of America
[ ] the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

VOGELSTEIN, Bert
3700 Breton Way
Baltimore, Maryland 21208
United States of America

This person is:

[ ] applicant only

[X] applicant and inventor

[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
[ ] all designated States
[X] the United States of America only
[ ] all designated States except the United States of America
[ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:
[X] agent [ ] common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

KAGAN, Sarah A.
BANNER & WITCOFF, LTD.
1001 G Street, N.W.
Eleventh Floor
Washington, D.C. 20001-4597
United States of America

Telephone No. (202) 508-9100

Facsimile No. (202) 508-9299

Teleprinter No. N/A

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KINZLER, Kenneth W.
1403 Halkirk Way
BelAir, Maryland 21015
United States of America

This person is:
[ ] applicant only
[X] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
[ ] all designated States
[X] the United States of America only
[ ] all designated States except the United States of America
[ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

YAN, Hai
4233 Hickory Avenue #C
Baltimore, Maryland 21211
United States of America

This person is:
[ ] applicant only
[X] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CN

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
[ ] all designated States
[X] the United States of America only
[ ] all designated States except the United States of America
[ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PAPADOPOULOS, Nicholas
90 Morningside Drive #2F
New York, NY 10027
United States of America

This person is:
[ ] applicant only
[X] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
[ ] all designated States
[X] the United States of America only
[ ] all designated States except the United States of America
[ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
[ ] applicant only
[ ] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
[ ] all designated States
[ ] the United States of America only
[ ] all designated States except the United States of America
[ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a)*(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

[X] **AP** **ARIPO Patent: GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **MZ** Mozambique, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

[X] **EA** **Eurasian Patent: AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

[X] **EP** **European Patent: AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

[X] **OA** **OAPI Patent: BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

[X] **AE** United Arab Emirates
[X] **AG** Antigua and Barbuda
[X] **AL** Albania .....
[X] **AM** Armenia .....
[X] **AT** Austria .....
[X] **AU** Australia .....
[X] **AZ** Azerbaijan
[X] **BA** Bosnia and Herzegovina .....
[X] **BB** Barbados
[X] **BG** Bulgaria .....
[X] **BR** Brazil .....
[X] **BY** Belarus .....
[X] **BZ** Belize
[X] **CA** Canada
[X] **CH and LI** Switzerland and Liechtenstein
[X] **CN** China .....
[X] **CR** Costa Rica .....
[X] **CU** Cuba .....
[X] **CZ** Czech Republic .....
[X] **DE** Germany .....
[X] **DK** Denmark .....
[X] **DM** Dominica
[X] **DZ** Algeria .....
[X] **EE** Estonia .....
[X] **ES** Spain .....
[X] **FI** Finland .....
[X] **GB** United Kingdom
[X] **GD** Grenada
[X] **GE** Georgia .....
[X] **GH** Ghana .....
[X] **GM** Gambia
[X] **HR** Croatia .....
[X] **HU** Hungary .....
[X] **ID** Indonesia
[X] **IL** Israel .....
[X] **IN** India .....
[X] **IS** Iceland
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Continuation of Box No. IV:

Continuation of Box No. V
US 60/158,160 filed 08 October 1999 (08.10.99)
US 09/461,047 filed 15 December 1999 (15.12.99)
US 09/504,860 filed 16 February 2000 (16.02.00)

ALTHERR, Robert F.
BANNER, Donald W.
BANNER, Mark T.
BANNER, Pamela I.
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PATHAK, Ajay S.
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All members of the firm of BANNER & WITCOFF, LTD. at the address, telephone and telefacsimile numbers as indicated in Box No. IV.

Box No. VI PRIORITY CLAIM [ ] Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 08 October 1999 (08.10.99)	60/158,160	US		
item (2) 15 December 1999 (15.12.99)	09/461,047	US		
item (3) 16 February 2000 (16.02.2000)	09/504,860	US		

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This international application contains the following **number of sheets**:

request	:	5 sheets
description (excluding sequence listing part)	:	18 sheets
claims	:	6 sheets
abstract	:	1 sheet
drawings	:	4 sheets
sequence listing part of description	:	0 sheet
Total number of sheets	:	34 sheets

This international application is **accompanied by** the item(s) marked below:

1. [X] fee calculation sheet (duplicate)
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4. [ ] statement explaining lack of signature
5. [ ] priority document(s) identified in Box No. VI as item(s):
6. [ ] translation of international application into (language):
7. [ ] separate indications concerning deposited microorganism or other biological material
8. [ ] nucleotide and/or amino acid sequence listing in computer readable form
9. [X] other *(specify)*: Transmittal

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

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Sarah A. Kagan
Agent for the Applicant(s)

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5. International Searching Authority (if two or more are competent): ISA/

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FEE CALCULATION SHEET
Annex to the Request

International application No.

Applicant's or agent's file reference 01107.00054

Date stamp of the receiving Office

Applicant
THE JOHNS HOPKINS UNIVERSITY

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE	240 | T	
2. SEARCH FEE	925 | S	

International search to be carried out by EP
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3. INTERNATIONAL FEE

Basic Fee
The international application contains 34 sheets.

first 30 sheets	b1 | 427	
4 X 10.00 = remaining sheets X additional amoun	b2 | 40	
Add amounts entered at b1 and b2 and enter total at B	467 | B	

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8 X 92.00 = number of designation fees X amount of designation fee payable (*maximum 8*)	736 | D	
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4. FEE FOR PRIORITY DOCUMENT *(if applicable)*	45 | P	
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Applicant(s): THE JOHNS HOPKINS UNIVERSITY

Title: CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

Filing Date: October 6, 2000

Client: THE JOHNS HOPKINS UNIVERSITY

Our Ref.: 01107.00054 Client Ref.: JM-3574

Attorney/Secretary: SAK/MH

Serial No.:

B&W Rev. 4/99

RO/US BOX PCT

PCT

B&W # 01107-00054 Atty/Sec SAK/MH Date OCTOBER 6, 2000 Inspected By: _____

In re International Application of THE JOHNS HOPKINS UNIVERSITY

International Application No. TBA

For CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

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- [X] Request: # of Sheet(s) 5
  - Replacement Sheet(s) [ ] YES [X] NO
- [X] Fee Calculation Sheet: Original + 1 copy
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  - Replacement Sheet(s) [ ] YES [X] NO
- [X] Claims: # of Sheet(s) 6
  - Replacement Sheet(s) [ ] YES [X] NO
- [X] Abstract: 1 Sheet
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CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

This invention was supported with U.S. government funds, NIH grants CA43460, CA57345, CA62924, CA67409, CA72851. The government therefore retains certain rights in the invention. This application claims the benefit of provisional application Serial No. 60/158,160 filed October 8, 1999, and is a continuation-in-part of patent application Serial No. 09/461,047 filed December 15, 1999.

BACKGROUND OF THE INVENTION

The problem with humans and other mammals, at least from a genetic diagnostic perspective, is that they are diploid. Mutations in one allele, such as those responsible for all dominantly inherited syndromes, are always accompanied by the wild-type sequence of the second allele. Though many powerful techniques for genetic diagnosis have been developed over the past decade, all are compromised by the presence of diploidy in the template. For example, the presence of a wild-type band of the same electrophoretic mobility as a mutant band can complicate interpretation of sequencing ladders, especially when the mutant band is of lower intensity. Deletions of a segment of DNA are even more problematic, as in such cases only the wild-type allele is amplified and analyzed by standard techniques. These issues present difficulties for the diagnosis of monogenic diseases and are even more problematic for multigenic diseases, where causative mutations can occur in any of several different genes. Such multigenism is the rule rather than the exception for common predisposition syndromes, such as those associated with breast and colon cancer, blindness, and hematologic, neurological, and cardiovascular diseases. The sensitivity of genetic diagnostics for these diseases is currently suboptimal, with 30% to 70% of

cases refractory to genetic analysis.

There is a need in the art for simply separating and analyzing individual alleles from human and other mammalian cells.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for detecting mutations in a gene of interest on a human or other mammalian chromosome.

It is another object of the invention to provide a method for making test cells suitable for sensitive genetic testing.

It is yet another object of the invention to provide a population of fused cell hybrids which are useful for genetic analysis.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment a method of detecting mutations in a gene of interest of a human or other mammal is provided. Cells of a human or other mammal are fused to rodent cell recipients to form human-rodent or other mammal-rodent cell hybrids. Fused cell hybrids are selected by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first human or other mammalian chromosome, forming a population of fused cell hybrids. A subset of hybrids are detected among the population of fused cell hybrids. The hybrids are haploid for a second human other mammalian chromosome which is not the same chromosome as the first human or other mammalian chromosome and which was not selected. The subset of hybrids are tested to detect a gene, an mRNA product of said gene, or a protein product of said gene. The gene resides on the second human or other mammalian chromosome. Diminished amounts of the mRNA or protein product or altered properties of the gene, mRNA, or protein product indicate the presence of a mutation in the gene in the human or other mammal.

According to another embodiment, a method is disclosed which provides test cells for genetic testing. The test cells are haploid for human or other mammalian genes. Cells of a human or other mammal are fused to transformed, diploid, rodent cell recipients to form human-rodent or other mammal-rodent cell hybrids. Fused cell hybrids are selected by selecting for a marker on each of a first human or other mammalian chromosome and a rodent chromosome, forming a population of cells

which stably maintain one or more human or other mammalian chromosomes in the absence of selection for the human or other mammalian chromosomes. Cells which are haploid for a second human or other mammalian chromosome which is distinct from the first human or other mammlian chromosome are detected among the population of cells; the second human or other mammalian chromosome was not selected.

Also provided by the present invention is a population of rodent-human or rodent-other mammalian hybrid cells wherein each homolog of at least 2 human or other mammalian autosomes is present in haploid form in at least one out of one hundred of the cells.

The present invention thus provides the art with a method which can be used to increase the sensitivity and effectiveness of various diagnostic and analytic methods by providing hybrid cells to analyze which are haploid for one or more genes of interest. The human or other mammalian chromosome content of the hybrid cells is stable and uniform.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Strategy for hybrid generation. The recipient mouse cell line E2 was fused with human lymphocytes and clones were subsequently selected with HAT plus geneticin, which kill unfused E2 cells and lymphocytes, respectively. All clones contained a human X chromosome responsible for growth in HAT. Clones were genotyped to determine which human chromosomes were retained. Chromosomes marked "A" and "B" represent the two homologs of a representative human chromosome. The average proportion of clones which retained neither, both, or either of the six chromosome homologs analyzed is indicated (see text). Mutational analysis was carried out on nucleic acids of clones which retained single alleles of the genes to be tested.

Fig. 2. Allelic status and gene expression in hybrids. **(Fig. 2A)** Polymorphic markers from the indicated chromosomes were used to determine the genotype of the indicated hybrids. "Donor" denotes the human lymphocytes used for fusion with the mouse recipient cells. **(Fig. 2B)** cDNA of E2 and four hybrids were used as templates to

amplify *hMSH2*, *hMSH6*, *hMLH1*, *hTGF β-RII*, *hPMS1*, *hPMS2*, and *APC* sequences. The results were concordant with the genotypes observed in **(Fig. 2A)**, in that hybrids 5 - 7 retained at least one allele of each of the chromosomes containing the tested genes, while hybrid 8 contained alleles of chromosomes 3, 5, and 7 but not of chromosome 2 (containing the *hMSH2, hPMS1, and hMSH6* genes).

Fig. 3. Mutational analysis of an HNPCC patient refractory to standard genetic diagnosis. Nucleic acids from the indicated hybrids were tested for retention of chromosomes 2 and 3 using polymorphic markers **(Fig. 3A)** and for expression of *hMSH2* and *hMLH1* genes on chromosomes 2 and 3, respectively **(Fig. 3B)**. Hybrids 1, 2, 3, and 6 contained allele A from chromosome 2 and did not express hMSH2 transcripts, while hybrids 4 and 5 contained the B allele and expressed hMSH2. hMLH1 expression served as a control for the integrity of the cDNA. **(Fig. 3C)** Sequences representing the indicated exons of *hMSH2* were amplified from the indicated hybrids. Exons 1- 6 were not present in the hybrids containing allele A, but exons 7 - 16 were present in hybrids containing either allele.

Fig. 4. Mutational analysis of Warthin family G. **(Fig. 4A)** Sequence analysis of RT-PCR products from hMSH2 transcripts of hybrid 1, containing the mutant allele of a Warthin family G patient, illustrates a 24 bp insertion (underlined; antisense primer used for sequencing). The wild-type sequence was found in hybrid 3, containing the wt allele. RT-PCR analysis of transcripts from lymphoid cells of the patient showed that the mutant transcript was expressed at significantly lower levels than the wild-type sequence. Sequence analysis of the genomic DNA of the same hybrids **(Fig. 4B)** showed that the insertion was due to a A to C mutation (antisense sequence, indicated in bold and underlined) at the splice acceptor site of exon 4, resulting in the use of a cryptic splice site 24 bp upstream. The signal of the mutant C is not as strong as the wild-type A in the donor's DNA. Such non-equivalence is not unusual in sequencing templates from diploid cells, and can result in difficulties in interpretation of the chromatograms. **(Fig. 4C)** Extracts from hybrids 1 and 5, carrying the mutant allele of chromosome 2, were devoid of hMSH2 protein, while extracts of hybrids 2 and 3, carrying the wt allele, contained hMSH2 protein. Hybrid 4 did not contain either allele of chromosome 2. Hybrids 1, 3, 4, and 5 each carried at least one allele of chromosome

3 and all synthesized hMLH1 protein. α-tubulin served as a protein loading control. Immunoblots with antibodies to the indicated proteins are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

We have devised a strategy for generating hybrids containing any desired human or other mammal's chromosome using a single fusion and selection condition. Importantly and unexpectedly, the human or other mamalian chromosomes in these hybrids were stable, and they expressed human or other mammalian genes at levels sufficient for detailed analysis. The approach is based on the principle that fusion between human or other mammal and rodent cells creates hybrid cells that contain the full rodent genomic complement but only a portion of the human or other mammalian chromosomes. In the past, selection for retention of a specific human or other mammalian chromosome (by complementation of an auxotrophic rodent cell, for example) has allowed the isolation of hybrids containing a desired chromosome (7, 8). Though such fusions have proven useful for a variety of purposes (8, 9), their utility has been limited by the availability of appropriate rodent recipients for many chromosomes and by the inefficiencies and variation of the fusion and selection conditions. For the analysis of multigenic diseases, it would be necessary to perform a separate fusion and selection for each chromosome.

The stability of the human or other mammalian chromosomes in the hybrids of the present invention was surprising. Though the human genetic constitution of radiation hybrids is relatively stable, this stability has been presumed to be due to the integration of small pieces of human DNA into rodent chromosomes following irradiation of the donor cells. The human chromosomes in whole cell fusions have been believed to be unstable unless continuous selection pressure for individual chromosomes was exerted. The reasons for the stability in our experiments is unclear, but may be related to the diploid nature of the rodent partner. Such diploidy reflects a chromosome stability that is unusual among transformed rodent cells. Previous experiments have indeed shown that chromosomally stable human cells retain all chromosomes upon fusion with other chromosomally stable human cells, unlike the situation when one of the two partners is chromosomally unstable.

The diploid, rodent recipient cells of the present invention provide useful

reagents for the facile creation of cells with functionally haploid human genomes. Nucleic acids or proteins from these hybrids can be used as reagents for any standard mutational assay. As mutational assays are constantly being improved and automated (1), the value of the hybrid-generated materials correspondingly increases. It may soon become possible, in fact, to examine the sequence of entire genes (promoters and introns in addition to exons). Nucleic acid templates generated from single alleles are clearly superior for such analyses, as the homogeneous nature of the templates dramatically enhances the signal to noise ratio of virtually any diagnostic assay. We therefore envision that this approach can be productively applied to a wide variety of research and clinical problems.

Genes of interest are typically those which have been found to be involved in inherited diseases. These include genes involved in colon cancer, breast cancer, Li-Fraumeni disease, cystic fibrosis, neurofibromatosis type 2, von Hippel-Lindau disease, as well as others. The identified genes include *APC*, *merlin*, *CF*, *VHL*, *hMSH2*, *p53*, *hPMS2*, *hMLH1*, *BRAC1*, as well as others. Mutations which can be identified at the protein level include those in sequences that regulate transcription or translation, nonsense mutations, splice site alterations, translocations, deletions, and insertions, or any other changes that result in substantial reduction of the full-length protein. Other subtler mutations can be detected at the nucleic acid level, such as by sequencing of RT-PCR products.

Cells of the human which may be used in fusions are any which can be readily fused to rodent cells. Peripheral blood lymphocytes (PBL) which are readily available clinical specimens are good fusion partners, with or without prior mitogenetic stimulation, whether used fresh or stored for over one year at -80° C. Since inherited mutations are the subject of the present method, any cells of the human body can be used, since all such cells contain essentially the same genetic complement. Cells of other mammals which can be used include in particular those of cats, dogs, cows, sheep, goats, horses, chimpanzees, baboons, and hogs. More generically, the cells of the other mammals can be selected from the ruminants, primates, carnivora, lagomorpha, and perissodactyla. Typically the other mammalian cell fusion partner is not a rodent cell.

Rodent cell recipients for fusion are preferably diploid, more preferably

oncogene-transformed, and even more preferably have microsatellite instability due to a defect in a mismatch repair gene. Selection of particular clones which grow robustly, are stably diploid, and fuse at a high rate is well within the skill of the ordinary artisan. The rodent cells may be, for example, from mice, rats, guinea pigs, or hamsters.

Fusion of cells according to the present invention can be accomplished according to any means known in the art. Known techniques for inducing fusion include polyethylene glycol-mediated fusion, Sendai virus-mediated fusion, and electro-fusion. Cells can desirably be mixed at a ratio of between 10:1 and 1:10 human to rodent. Clones of fused cells generally become visible after about two to three weeks of growth.

Fused hybrid cells can be selected using any markers which result in a positively selectable phenotype. These include antibiotic resistance genes, toxic metabolite resistance genes, prototrophic markers, etc. The surprising advantage of the present invention is that a single marker on a single human or other mammalian chromosome can be used in the selection, and that stable hybrids containing more than just the single, selected human or other mammalian chromosome result. Thus markers on other chromosomes can be analyzed even when the chromosomes on which the markers reside were not selected.

Fused hybrid cells can be analyzed to determine that they do in fact carry a human or other mammalian (non-rodent) chromosome which carries a gene of interest. Hybrid cells which have either of the two relevant human or other mammalian chromosomes can be distinguished from each other as well as from hybrids which contain both of the two human or other mammalian chromosomes. See **Fig. 1.** While any means known in the art for identifying the human or other mammalian chromosomes can be used, a facile analysis can be performed by assessing microsatellite markers on the human or other mammalian chromosome. Other linked polymorphic markers can be used to identify a desired human or other mammalian chromosome in the hybrids.

Once hybrid cells are isolated which contain one copy of a human or other mammalian gene of interest from a human or other mammal who is being tested, mutation analysis can be performed on the hybrid cells. The genes can be tested directly for mutations, or alternatively the mRNA or protein products of the genes can

be tested. Mutations that result in reduced expression of the full-length gene product should be detectable by Western blotting using appropriate antibodies. Tests which rely on the function of the protein encoded by the gene of interest and enzyme assays can also be performed to detect mutations. Other immunological techniques can also be employed, as are known in the art.

If an immunological method is used to detect the protein product of the gene of interest in the hybrids, it is desirable that antibodies be used that do not cross-react with rodent proteins. Alternatively, the rodent genes which are homologous to the gene of interest can be inactivated by mutation to simplify the analysis of protein products. Such mutations can be achieved by targeted mutagenesis methods, as is well known in the art.

Functional tests can also be used to assess the normalcy of each allelic product. For example, if one inserted an expression construct comprising a β-galactosidase gene downstream from a p53 transcriptional activation site, into a rodent-human hybrid cell that contained human chromosome 17 but no endogenous p53, then one could detect mutations of the p53 on the human chromosome 17 by staining clones with X-gal. Other enzymatic or functional assays can be designed specifically tailored to the gene of interest.

Any method of detecting mutations at the DNA or RNA level as are known in the art may be employed. These include without limitation, sequencing, allele-specific PCR, allele-specific hybridization, microarrays, DGGE, and automated sequencing.

It is a possibility that expression of the gene of interest might be inhibited in the hybrid cell environment. In order for the loss of expression of a gene of interest in the hybrid cells to be meaningfully interpreted as indicating a mutation in the human or other mammal, one must confirm that the gene of interest, when wild-type, is expressed in rodent-human or other mammal hybrid cells. This confirmation need not be done for each patient, but can be done once when the assay is being established.

When the assay of the present invention indicates that a mutation exists in the gene of interest, other family members can be tested to ascertain whether they too carry the mutation. Alternatively, the other family members can be tested to see if they carry the same chromosome as the affected family member. This can be determined by testing for a haplotype, *i.e.*, a set of distinctive markers which are found on the

chromosome carrying the mutation in the affected family member. Determination of a haplotype is a by-product of performing the assay of the invention on the first family member. When the hybrid cells are tested to confirm the presence of the relevant chromosome in the hybrid, for example by use of microsatellite markers, a distinctive marker set will be identified, which can then be used as a haplotype.

Mixed populations of hybrid cells made by the fusion process of the present invention may contain hybrid cells which are haploid for a number of different human or other mammalian chromosomes. Typically each homolog of at least 2, at least 5, at least 10, at least 15, at least 20, or even 22 human or other mammalian autosomes will be present in the population in a haploid condition in at least one out of one hundred, seventy-five, fifty, thirty or twenty-eight of the cells. Thus a high proportion of the cells contain multiple human or other mammalian chromosomes, and a relatively small number of cells must be tested to find cells harboring a single copy of a non-selected chromosome.

Populations of cells resulting from a single hybrid are uniform and homogeneous due to the high stability of the human or other mammalian chromosomes in the hybrid cells of the invention. Thus at least 75%, 80%, 85%, 90%, 95%, 97%, 99%, or 100% of the cells in the population resulting from a single hybrid cell contain the same complement of human or other mammalian chromosomes.

The following examples provide experimental details which demonstrate one of many ways to carry out the invention. The invention is not limited to the particular methods of cells employed in the examples. The claims and the specification as a whole provide the measure of the invention.

Examples

Example 1

An outline of the approach is presented in Fig. 1. The rodent fusion partner was a line derived from mouse embryonic fibroblasts transformed with ras and adenovirus E1A oncogenes. HPRT-deficient subclones of this line were generated, and one subclone (E2) was chosen for further experimentation based on its robust growth characteristics, maintenance of diploidy, and fusion efficiency (10). Human lymphocytes cells were mixed with E2 cells at an optimum ratio and electrofused, and hybrids selected in geneticin (to kill unfused human cells) and HAT (to kill unfused E2

cells) (11). Colonies appearing after two weeks of growth were expanded and RNA and DNA prepared for analysis. From a single fusion experiment, an average of 36 hybrid clones were obtained (range of 17 to 80 in five different individuals).

All hybrids contained the human X chromosome, as this chromosome contains the HPRT gene allowing growth in HAT. To determine whether other human chromosomes were present in the hybrids, polymorphic microsatellite markers (12) were used as probes in PCR-based assays (Fig. 2A). We focused on the chromosome arms (2p, 2q, 3p, 5q, 7q, and 16q) known to contain colorectal cancer (CRC) predisposition genes. One copy of each of these chromosome arms was present in a significant fraction of the hybrid clones. For example, of 476 hybrids derived from 14 individuals and examined for chromosome 3, 136 hybrids contained neither donor chromosome, 211 hybrids contained both donor chromosomes, 60 hybrids contained one parent's chromosome, and 69 hybrids contained the other parent's chromosome. Similar retention frequencies were found for all six chromosome arms analyzed. Testing of markers from both arms of a single chromosome showed that whole chromosomes, rather than chromosome fragments, were generally retained in the hybrids. This result was confirmed with fluorescence in situ hybridization (FISH) on metaphase spreads from the hybrids, which indicated the presence of 11 ±3 human chromosomes in each hybrid cell. Calculations based on the genotypic data indicated that the analysis of 25 hybrids would ensure a 95% probability of identifying at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of a single chromosome under study. Moreover, it would require only 45 hybrids to similarly ensure that each allele of all 22 autosomes was present and separated from its homolog in at least one hybrid (13).

Example 2

Two other features of the hybrids were essential for the analyses described below. First, the human chromosome complements of the hybrids were remarkably stable. Polymorphic marker analysis in ten hybrids revealed identical patterns of retention after growth for 90 (30 passages) generations after initial genotyping. Second, those hybrids containing the relevant chromosome expressed every human gene assessed, including all known colorectal cancer susceptibility genes (the *hMSH2*

and *hMSH6* genes on chromosome 2p, the *hPMS1* gene on chromosome 2q, the *TGF-β Receptor Type II* gene and *hMLH1* gene on chromosome 3p, the *APC* gene on chromosome 5q, the *hPMS2* gene on chromosome 7q, and the *E-cadherin* gene on chromosome 16q; representative examples in Fig. 2B) (14).

Example 3

Having established the stability and expression patterns of CRC-predisposition genes in these hybrids, we used this "conversion" approach to investigate ten patients who had proven refractory to standard genetic diagnostic techniques. Each of these patients had a significant family history of colorectal cancer and evidence of mismatch repair deficiency in their tumors, yet sequencing of the entire coding sequence of each known MMR gene had failed to reveal mutations. Indeed, these and similar studies have prompted the speculation that other major HNPCC genes must exist. (25-34) Hybrids were generated from lymphocytes of each patient, and at least one hybrid containing the maternal allele and one hybrid containing the paternal allele of each MMR gene was isolated. Analysis of the nucleic acids from these hybrids revealed specific mutations in all ten patients (Table 1). In every case, an abnormality was found in a single allele of either *hMSH2* or *hMLH1*. The nature of the abnormalities revealed why they had not been detected with the standard methods previously used for their analysis. Three cases were due to large deletions, encompassing six or seven exons. When corresponding nucleic acids from the cells of such patients are evaluated by any PCR-based method, only the wild type sequences from the unaffected parent would be amplified, leading to the false impression of normalcy (for example, case #1 in Figure 3). Though Southern blotting can reveal deletions of one or a few exons in MMR, larger deletions are refractory to such blotting methods. In three cases (#4, 6, and 9), no transcript was generated from one allele, though the sequences of all exons and intron-exon borders from this allele were normal. Presumably, mutations deep within an intron or within the promoter of the gene were responsible. The absence of transcripts from one specific allele of these three patients was confirmed in at least three other converted hybrids from each patient. In four other cases, point mutations were found (Table 1). These mutations were not detected in the original sequence analyses because the signals from the mutant allele were not as robust as those from the wild type. Such asymmetry can be caused by instability of mutant transcripts due to

nonsense mediated decay (36-38), or to nucleotide preferences of the polymerases in specific sequence contexts, and represents a common problem for both manual and automated sequencing methods (39). The conversion approach eliminates these problems because only one sequence can possibly be present at each position. A good example of this was provided by Warthin G (17). The mutation in this prototype kindred was an A to C transversion at a splice site. The signal from the mutant "C" in the sequencing ladder was not as intense as the wild type "A" (Fig. 4b). This mutation led to the use of a cryptic splice site 24 bp upstream of exon 4, and an under-represented transcript with a 24 base insertion (Fig. 4a). To demonstrate that this mutation had an effect at the protein level, we analyzed the hybrids by immunoblotting with specific antibodies (19). The hybrids containing the mutant allele did not make detectable levels of human hMSH2 protein, though they did synthesize normal levels of a control protein (Fig. 4C).

The results described above demonstrate that individual alleles of human chromosomes can be readily isolated upon fusion to mouse cells.

HNPCC provides a cogent demonstration of the power of the conversion approach because it is a common genetic disease that has been widely studied. In the last three years, for example, extensive analyses of the major MMR genes have been performed in 303 HNPCC kindreds from nine cohorts distributed throughout the world (25-34). Based on the fraction of such patients with characteristic microsatellite instability in their cancers (30-34), it can be estimated that 239 (78%) of the kindreds had germ-line mutations of mismatch repair genes. Yet MMR gene mutations were identified in only 127 (42%) of these 239 kindreds (25-34). Our cohort was similar, in that it was derived from a total of 25 kindreds, 22 of whom had tumors with microsatellite instability and presumptive MMR gene mutations. Of these 22, our initial analyses revealed mutations in only 12 (54%) (ref. 14 and unpublished data). Mutations of the other ten patients were only revealed upon conversion analysis, which thereby increased the sensitivity from 54% to 100%. The conclusion that virtually all cases of HNPCC associated with MSI are due to germline mutations of known MMR genes is consistent with recent immunohistochemical data demonstrating the absence of either MSH2 or MLH1 protein staining in the cancers from the great majority of HNPCC patients (40, 41). A corollary of these results is that the search for new human

MMR genes should not based on the premise that a large fraction of HNPCC cases will prove attributable to such unknown genes.

The system described above can be applied to other genetic diseases in a straight forward manner. It should be emphasized that this approach is not a substitute for the many powerful methods currently available to search for specific mutations. Rather, conversion can be used to maximize the sensitivity of existing techniques. Converted nucleic acids provide the preferred substrates for such methods because of the higher signal to noise attainable and the inability of the wild type allele to mask or confound detection of the mutant allele. As DNA-based mutational assays are improved in the future, and progressive incorporate microarrays and other automatable features (42-44), the value of conversion-generated nucleic acids will correspondingly increase, significantly enhancing the effectiveness of genetic tests for hereditary disease.

Methods

Cell culture

Mouse embryonic fibroblasts were derived from MSH2-deficient mice (46) and transformed with adenovirus E1A and RAS oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

Cell fusion and the generation of hybrids

The patients were from kindreds with HNPCC as defined by the Amsterdam criteria (44); in no case was linkage analysis feasible due to the lack of a sufficient number of affected individuals. Microsatellite instability (MSI) in the cancers from these patients was determined through the markers recommended in ref. 45. 3 x 10^6 E2 cells and 12 x 10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 μl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX ElectroCell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 μsec each. The cells from one

fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersburg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. From a single fusion, an average of 23 +/- 15 hybrid clones were obtained. The lymphocytes used for the experiments described here were derived from Epstein-Barr Virus infection of peripheral blood leukocytes, but it was found that freshly drawn lymphocytes could also be successfully fused and analyzed using identical methods.

Genotyping

Genotyping was performed as described (12). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788 and D2S1360, D2S1384, D3S2406, D7S1824, and D16S3095, from chromosome 2p, 2q, 3p, 5q, 7q and 16q, respectively. Fluorescence in situ hybridization was performed as described previously (21).

PCR and sequencing

Polyadenylated RNA was purified and RT-PCR performed as described previously. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through an internet site.

Statistical analysis

the number of hybrids containing none, both or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the number of hybrids required to generate mono-allelic hybrids containing specific numbers of each chromosomes.

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10. E2 cells were derived from mouse embryonic fibroblasts derived from MSH2-deficient mice (generously provided by T. Mak) and transformed with adenovirus *E1A* and *RAS* oncogenes. HPRT-deficient subclones were selected by growing the fibroblasts in 10 μM 2-amino-6-mercaptopurine. Clones were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS and 10 μM 2-amino-6-mercaptopurine.

11. 3 x 10^6 lymphocytes cells were mixed, washed, and centrifuged twice in fusion

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[1]All references are explicitly incorporated by reference.

medium (0.25 M D-sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% Bovine Serum Albumin (BSA), pH 7) and resuspended in 640 µl fusion medium. The solution was pipetted into a cuvette (BTX cuvette electrode 470; BTX, San Diego). Cells were fused using a BTX Electro Cell Manipulator, model ECM200. The settings that yielded the greatest number of hybrids were: 30V (AC) for 22 seconds, followed by three 300V (DC) pulses of 15 µsec each. The cells from one fusion were plated into three 48-well plates (Costar) in DMEM supplemented with 10% FCS. After 24 hours, the medium was replaced by DMEM supplemented with 10% FCS, 0.5 mg/ml geneticin and 1 x HAT (Life Technologies, Gaithersberg, MD). The medium was changed after a week. Hybrid clones became visible two weeks after fusion and were expanded for another week prior to genotyping. The lymphocytes used for the experiments described here were derived from Epstein-Barr virus infection of peripheral blood leukocytes, but we found that freshly drawn lymphocytes could also be successfuly fused and analyzed using identical methods

12. Genotyping was performed as described in F.S. Leach et al., *Cell* 75, 1215 (1993). PCR products were separated on 6% denaturing gels and visualized by autoradiography. The microsatellite markers used were D2S1788, D2S13360, D3S2406, D7S1824, and D16S3095, from chromosomes 2p, 2q, 3p, 5q, and 16q, respectively.

13. The numbers of hybrids containing none, both, or a single allele of each chromosome tested were consistent with a multinomial distribution. Monte Carlo simulations were used to estimate the numbers of hybrids required to generate mono-allelic hybrids containing specific numbers of chromosomes.

14. Polyadenylated RNA was purified and RT-PCR performed as described in B. Liu et al., *Nat Medicine* **2**, 169 (1996).

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18. Sequencing was performed using ABI Big Dye terminators and an ABI 377 automated sequencer. All primers used for amplification and sequencing will be made available through a *Science* internet site.

19. Cytoplasmic extracts of cells hybrids were separated by electrophoresis through SDS-polyacrylamide gels and immunoblotted with antibodies specific for human hMSH2 (#NA26, Calboiochem), human hMLH1 (#13271A, Pharmingen), or β-Tubulin (#N357, Amersham).

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CLAIMS

1. A method of detecting mutations in a gene of interest of a non-rodent mammal, comprising the steps of:

fusing cells of a non-rodent mammal to rodent cell recipients to form non-rodent mammal-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a first marker contained on a rodent chromosome and for a second marker contained on a first non-rodent mammalian chromosome, to form a population of fused cell hybrids;

detecting among the population of fused cell hybrids a subset of hybrids which are haploid for a second non-rodent mammalian chromosome which is not the same chromosome as the first non-rodent mammalian chromosome and which was not selected;

analyzing said subset of hybrids to detect a mutation in an mRNA product of a gene or in said gene, wherein the gene resides on the second non-rodent mammalian chromosome.

2. The method of claim 1 wherein the rodent cell is diploid.

3. The method of claim 1 wherein the rodent cell recipients are deficient in mismatch repair.

4. The method of claim 1 wherein the rodent cell recipient is geneticin resistant.

5. The method of claim 1 wherein the rodent cell recipients are transformed with an oncogene.

6. The method of claim 5 wherein the oncogene is *ras*.

7. The method of claim 5 wherein the oncogene is *E1A*.

8. The method of claim 1 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

9. The method of claim 1 wherein the rodent cell recipients are *MSH2*$^-$.

10. The method of claim 1 wherein the rodent cell recipient is diploid, and contains both a selectable marker and a counterselectable marker.

11. The method of claim 10 wherein the counterselectable marker is HPRT deficiency.

12. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third non-rodent mammalian chromosome, wherein the first, second, and third non-rodent mammalian chromosomes are distinct, and neither the second nor the third non-rodent mammal chromsomes were selected.

13. The method of claim 1 further comprising the step of:

detecting among the population of fused cell hybrids a third and fourth non-rodent mammalian chromosome, wherein the first through fourth non-rodent mammalian chromosomes are distinct, and neither the second through fourth non-rodent mammal chromsomes were selected.

14. The method of claim 12 further comprising the step of testing said subset of hybrids to detect an mRNA product of a second gene or a protein product of said second gene, wherein the second gene resides on the third non-rodent mammalian chromosome, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product from said second gene indicate the presence of a mutation in the second gene in the non-rodent mammal.

15. The method of claim 13 testing said subset of hybrids to detect an mRNA product of a second and third gene or a protein product of said second and third gene, wherein the second and third genes reside on the third and fourth non-rodent mammalian chromosomes, wherein diminished amounts of said mRNA or protein product or altered properties of said mRNA or protein product of said third or fourth genes indicate the presence of a mutation in third or fourth genes in the non-rodent mammal.

16. The method of claim 1 wherein the cells of the non-rodent mammal are lymphocytes.

17. The method of claim 1 wherein the step of detecting a subset of hybrids which are haploid for the second non-rodent mammalian chromosome is accomplished by identifying the presence of a microsatellite marker on the second non-rodent mammalian chromosome.

18. The method of claim 1 wherein the step of analyzing to detect a mutation is performed by a technique selected from the group consisting of: nucleic acid sequencing, allele-specific PCR, allele-specific hybridization, hybridization to a

microarray, discontinuous gradient gel electrohoresis (DGGE), and automated nucleic acid sequencing.

19. A method of providing test cells for genetic testing, wherein said test cells are haploid for genes of a non-rodent mammal, comprising the steps of:

fusing cells of a non-rodent mammal to transformed, diploid, rodent cell recipients to form non-rodent mammal-rodent cell hybrids;

selecting for fused cell hybrids by selecting for a marker on each of a first non-rodent mammalian chromosome and a rodent chromosome, whereby a population of cells are formed which stably maintain one or more non-rodent mammalian chromosomes in the absence of selection for the non-rodent mammalian chromosomes;

detecting among the population of cells those cells which are haploid for a second and third non-rodent mammalian chromosome which are distinct from the first non-rodent mammalian chromosome and which were not selected.

20. The method of claim 19 wherein the rodent cell recipient is geneticin resistant.

21. The method of claim 19 wherein the rodent cell recipients are transformed with an oncogene.

22. The method of claim 21 wherein the oncogene is *ras*.

23. The method of claim 21 wherein the oncogene is *E1A*.

24. The method of claim 19 wherein the rodent cell recipients are transformed with *ras* and *E1A* oncogenes.

25. The method of claim 19 wherein the rodent cell recipients are deficient in mismatch repair.

26. The method of claim 19 wherein the rodent cell recipient contains a counterselectable marker.

27. The method of claim 26 wherein the counterselectable marker is HPRT deficiency.

28. The method of claim 19 wherein the cells of the non-rodent mammal are lymphocytes.

29. The method of claim 19 wherein the step of detecting cells which are haploid for said second and third non-rodent mammalian chromosomes is performed

by identifying the presence of a microsatellite marker on said second and third non-rodent mammalian chromosomes.

30. The method of claim 19 further comprising the step of:

detecting among the population of cells, cells haploid for a fourth non-rodent mammalian chromosome which is distinct from the first, second, and third non-rodent mammalian chromosomes and which was not selected.

31. The method of claim 19 futher comprising the step of:

detecting among the population of cells, cells haploid for a fourth, fifth, and sixth non-rodent mammalian chromosome, wherein said fourth through sixth non-rodent mammalian chromosomes are distinct from the first, second and third non-rodent mammalian chromosomes and were not selected.

32. The method of claim 19 further comprising:

testing nucleic acids of a cell haploid for the third non-rodent mammalian chromosome for a mutation in a gene on the third non-rodent mammalian chromosome.

33. The method of claim 19 further comprising:

testing proteins of a cell haploid for the second and third non-rodent mammalian chromosome for a mutation in a gene on each of the second and third non-rodent mammalian chromosomes.

34. The method of claim 1 wherein mRNA of a cell in the subset of hybrids is tested for a mutation in a gene on the second non-rodent mammalian chromosome.

35. The method of claim 1 wherein proteins of a cell in the subset of hybrids are tested for a mutation in a gene on the second non-rodent mammalian chromosome.

36. A population of rodent-non-rodent mammal hybrid cells wherein each homolog of at least 2 non-rodent mammal autosomes is present in haploid form in at least one out of one hundred of the cells.

37. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

38. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

39. The population of claim 36 wherein each homolog of at least 5 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

40. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

41. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

42. The population of claim 36 wherein each homolog of at least 10 non-rodent mammal autosomes is present in haploid form in at least one out of a thirty of the cells.

43. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

44. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

45. The population of claim 36 wherein each homolog of at least 15 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

46. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred of the cells.

47. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

48. The population of claim 36 wherein each homolog of at least 20 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

49. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of a hundred

of the cells.

50. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of fifty of the cells.

51. The population of claim 36 wherein each homolog of at least 22 non-rodent mammal autosomes is present in haploid form in at least one out of thirty of the cells.

52. A population of rodent-non-rodent mammal hybrid cells which stably maintain their non-rodent mammalian chromosome content such that at least 95% of the hybrid cells contain the same non-rodent mammalian chromosomes.

53. The population of rodent-non-rodent mammal hybrid cells of claim 52 whererein at least 97% of the hybrid cells contain the same non-rodent mammalian chromosomes.

54. The population of rodent-non-rodent mammal hybrid cells of claim 52 whererein at least 99% of the hybrid cells contain the same non-rodent mammalian chromosomes.

55. The method of claim 1 wherein the non-rodent mammal is a human.

56. The method of claim 1 wherein the non-rodent mammal is selected from the group consisting of goats, sheep, horse, cows, pigs, hogs, cats, and dogs.

CONVERTING DIPLOIDY TO HAPLOIDY FOR GENETIC DIAGNOSIS

ABSTRACT OF THE DISCLOSURE

Detection of mutations associated with hereditary diseases is complicated by the diploid nature of mammalian cells. Mutations present in one allele are often masked by the wild-type sequence of the other allele. Individual alleles can be isolated from every chromosome within somatic cell hybrids generated from a single fusion. Nucleic acids from the hybrids can be analyzed for mutations in an unambiguous manner. This approach was used to detect two cancer-causing mutations that had previously defied genetic diagnosis. One of the families studied, Warthin Family G, was the first kindred with a hereditary colon cancer syndrome described in the biomedical literature.